Causes and Consequences of Cooperative Construction in the Mice Mus spicilegus and Peromyscus polionotus

Causes and consequences of cooperative construction in the mice *Mus spicilegus* and *Peromyscus polionotus*

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ABSTRACT

The cooperative construction of shared dwellings is a phylogenetically-widespread evolutionary puzzle. Shared shelters are common goods – all individuals in the shelter benefit, at the expense of those individuals that contribute to the construction. The evolution of cooperation requires existing variation for selection to act upon and genetic benefits to cooperators, through inclusive fitness or direct rewards. This study focuses on two genera of mice, *Mus* and *Peromyscus*, to examine shared construction and social monogamy as potential transitions to more sophisticated forms of sociality, such as cooperative breeding.

The mound-building mouse (*Mus spicilegus*) is named for the large mounds that groups of mice build and beneath which they overwinter. Variation in mtDNA and 14 microsatellites show limited genetic structure across the geographic range of *M. spicilegus*. Mice from the same mound are more genetically related than mice from different mounds, and males and females associated with a mound are equally likely to be relatives. However in spring, when breeding begins, male kin are more likely to share a territory than are female kin. One possible interpretation is that males associate with kin to minimize the costs of being cuckolded, as this study finds evidence of multiple paternity in every litter genotyped. By increasing the chances of the cuckold being a brother, a male still gains

inclusive fitness benefits from paternal care to extra-pair offspring in this socially monogamous species.

Behavioral experiments show that another socially monogamous mouse species, the oldfield mouse (*Peromyscus polionotus*), can coordinate construction with unfamiliar, unrelated conspecifics. In contrast, two other closely related *Peromyscus* species do not dig longer burrows in pairs than they would have as individuals. Male-female *P. polionotus* pairs tend to dig longer burrows than pairs of the same sex, but males within opposite sex pairs do most of the digging, particularly when paired with an unfamiliar female. Male burrowing could be the product of female choice in this monogamous species. In *M. spicilegus* and *P. polionotus*, shared parental care and construction shed light on the evolution of cooperation and conflict.

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INTRODUCTION

Communal construction is fascinating because of the complexity of both social and architectural behaviors. In his chapter on instinct in the *Origin,* Darwin (1859) argues that elaborate behaviors, just like structures of bewildering intricacy, are products of natural selection. Consequently, one need only look to related organisms for intermediate and less elaborate examples to shed light on the process of evolving complex adaptations, be they eyes or beehives. Since then, several studies have combined the same logic with molecular phylogenetic methods to show that animal architecture does indeed evolve in stages (Winkler & Sheldon 1993; Kusmierski et al. 1997; Eberhard 1998; Zyskowski & Prum 1999; Weber & Hoekstra 2009).

The origin and maintenance of cooperative behavior is a subsequent evolutionary puzzle that Darwin did not fully appreciate in the absence of a good model of inheritance. If genes are less likely to be inherited when their carriers make sacrifices for the benefit of others in the gene pool, how does selection increase the frequency of genes for altruism (Williams 1966)? There are several answers to this question, many of which apply to mutualisms between species, but this introduction will focus on cooperation within species. An elegant solution is for benefactors and recipients to share genes for altruistic actions through recent common descent, so that what an altruistic individual loses in direct fitness is offset by her inclusive fitness through the reproductive success of her relatives (Hamilton 1964). A similar notion from a gene's-eye view, is the positive assortment of cooperators (Queller 1992, 1994; Fletcher & Doebeli 2009). However, cooperation does not always occur between genetic relatives. A second solution is reciprocal altruism, where repeated interactions and mutual policing ensure that both parties benefit from cooperating rather than trying to cheat

(Trivers 1971; Axelrod & Hamilton 1981). A third possibility is to broaden the definition of cooperation to encompass situations also known as by-product mutualisms (West-Eberhard 1975; Mesterton-Gibbons & Dugatkin 1992), when all agents are united against a common threat, and cheaters at a sufficiently high frequency are likely to do worse than they would by cooperating. For instance, genetically different tumor cells can benefit from releasing diffusible substances to combat host defenses that neither tumor cell line could survive alone (Axelrod et al. 2006).

A similar situation is formalized for two players in the hawk-dove game (Maynard Smith & Price 1973; Maynard Smith 1982), also known as a game of chicken, or a snowdrift game (Doebeli & Hauert 2005). In contrast to the classic prisoners dilemma, where the only stable equilibrium is mutual defection, a snowdrift game converges to a mixed stable equilibrium of both cooperators and defectors because cooperation and defection are both rewarded even when rare. The analogy of two people stuck in a snowdrift illustrates how the payoff for mutual defection (neither person shoveling snow) is lower than the payoff for a cooperator who shovels snow for a defector who remains warm in his car. One is better off performing the cooperative action of clearing the road than freezing in one's car out of spite, just because the other person also refuses to get out and shovel, but the defector who stays warm while another shovels does better than mutual cooperators.

The N-player version of a snowdrift game, the volunteer's dilemma, is well suited to many situations requiring cooperation in nature (Archetti 2009; Archetti et al. 2011), where populations are likely to reach a stable equilibrium with both cooperators and cheaters present. Most interestingly, the volunteer's dilemma does not require relatedness for

cooperation to exist, and the dilemma occurs because the more individuals there are that could contribute to the common good, the lower the probability of any individual volunteering. Thus a plausible way to increase cooperation is to raise the cost to the group if the public good is not produced.

Communal construction can be viewed as a volunteer's dilemma (Archetti 2009). Animal structures are a common good, but they take energy and time to build, constituting a substantial reproductive opportunity cost. Nevertheless, there are many examples of communal structures, from sociable weaver's condominium-style nests, to beaver lodges, to termite mounds, so their evolution demands explanation. For many of these structures, particularly those of the hymenoptera, inclusive fitness benefits to the colony provide an ample ultimate explanation for the evolution of cooperative construction. Indeed, these physical objects can be viewed as extended phenotypes of the genes within organisms building and benefiting from their architectural efforts (Dawkins 1982). However in other cases, unrelated individuals, or even members of different species will share a shelter. In these cases, an ultimate explanation for the origin and maintenance of communal construction seems consistent with a volunteer's dilemma. Reciprocal altruism requires individuals to keep track of who is contributing, and is less likely to apply to a broad range of organisms.

Sharing a shelter could be a common precursor to more sophisticated forms of sociality and cooperative breeding (Costa 2006). The notion is that a shelter constitutes a shared and defensible resource, thereby providing an opportunity for group living to evolve under favorable ecological conditions. With more than one individual to build or guard the shelter,

there can be selection for specialization and a division of labor. A division of labor is best exemplified by eusocial insects, and some of their solitary relatives, such as halictine bees, will divide the tasks of nest construction when experimentally forced into pairs (Jeanson et al. 2005). Arguably, incipient forms of cooperation such as a division of labor or communal living in the absence of cooperative breeding are the best places to look for both ultimate and proximate causes of complex sociality.

As all extended phenotypes, from social interactions to physical structures have the potential to alter the selective environment, looking at the consequences of communal construction are also important for understanding the historic trajectories that lineages with more complex sociality could have taken. For instance, social systems and shared resources like large nests can alter the dispersal patterns of a species, which can, in turn, alter the genetic composition of populations over space and time. The first part of this dissertation will focus on the mound-building mouse (*Mus spicilegus*) that lives communally during part of the year, and engages in the cooperative construction of shelters, but does not breed in groups. Chapter one introduces this relatively obscure relative of the laboratory mouse (*Mus musculus*), and its evolutionary relationship to the rest of the *Mus* genus. Chapter two explores the consequences of gathering seasonally to construct mounds on the population structure and phylogeographic history of this species. Chapter three investigates the social and mating systems and dispersal patterns in *M. spicilegus*, to shed light on some of the ultimate factors driving the evolution of communal mound building. To investigate the proximate causes of cooperative construction, which are difficult to study in wild mice, chapter four focuses on paired burrowing in laboratory populations of North American mice in the genus *Peromyscus*, to see if rodent species that are usually solitary can display rudimentary forms of cooperative construction.

CHAPTER 1

Natural history of the mound-building mouse (*Mus spicilegus*)

While the house mouse (*Mus musculus*) is one of the workhorses of genetics and neurobiology, many of its closest relatives remain poorly understood (Auffray & Britton-Davidian 2012). There are several reasons for expanding our knowledge of other *Mus* species; one is to determine if the complex traits so often studied in laboratory mice are the result of common ancestry or convergence. Knowing the evolutionary history and ecology of *M. musculus* and its relatives provides a context for understanding the ultimate factors that shaped proximate mechanisms and traits discovered in the laboratory. Another reason for focusing on wild *Mus*, is that the wide distribution and breadth of habitats occupied by members of this genus, coupled with genetic, genomic and neurobiological tools from laboratory mice makes this an excellent system for studying the proximate mechanisms underlying a wide range of ecological adaptations, from social systems to cold tolerance. However, such broad comparisons will require a better understanding of the basic ecology and behavior of wild *Mus*, and their recent evolutionary histories. The next two chapters of this thesis will present new evidence on the phylogeography, population and kinship structure of the mound-building mouse (*Mus spicilegus*). The rest of this chapter will briefly introduce the *Mus* genus and subgenus before reviewing existing research on the evolution, ecology and behavior of *M. spicilegus*.

The *Mus* genus

The genus *Mus* diverged from other murines about 8-10mya, and currently comprises 41 described species (Auffray & Britton-Davidian 2012). These species are classified into four

monophyletic subgenera (Chevret et al. 2005; Veyrunes et al. 2006): *Coelomys* (Shrew mice) from Asia, *Nannomys* (African Pygmy mice) from sub-Saharan Africa, *Pyromys* (Spiny mice) from Asia and the famous *Mus* subgenus (Fig. 1.1) (Tucker 2007). Possibly because of a very rapid evolutionary radiation, the relationships between these subgenera were only recently resolved by chromosomal painting (Veyrunes et al. 2006).

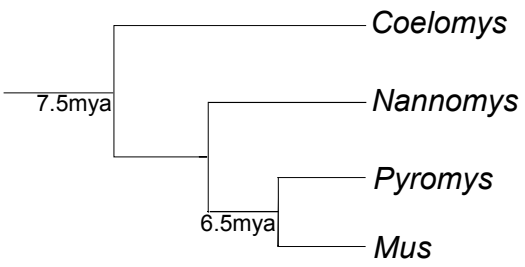


Figure 1.1 Showing relationships within the four subgenera in the *Mus* genus modified from (Veyrunes et al. 2006).

Species in the *Mus* subgenus are monophyletic and probably originated in Asia 2-3mya (Suzuki et al. 2004). The subgenus is further divided into two monophyletic clades—an Asian clade and a Palearctic clade. The latter comprises both the commensal house mouse (*Mus musculus*) and several field mice including the Algerian or Western Mediterranean mouse (*Mus spretus)*, the Macedonian or Balkan short-tailed mouse (*Mus macedonicus*) and the mound-building mouse (*Mus spicilegus*) (Tucker 2007). The exact phylogeny of the Palearctic clade remains unresolved because of the position of *M. spretus* (Fig. 1.2) (Tucker 2007; Suzuki & Aplin 2012).

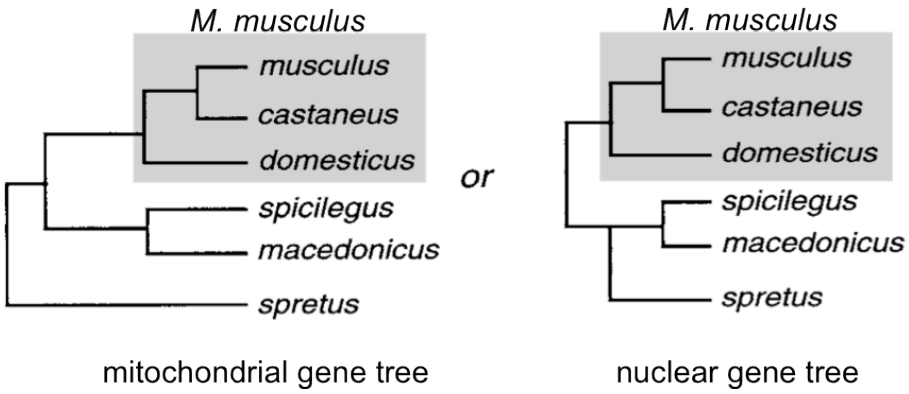


Figure 1.2 Two alternative phylogenies of the Palearctic clade of the *Mus* subgenus. The *Mus musculus* subspecies are enclosed in grey. On the left, the mitochondrial phylogeny places *M. spretus* basal to the other Palearctic *Mus* species. On the right, nuclear genes place *M. spretus* as sister to the *M. spicilegus*/ *M. macedonicus* clade. Modified from (Tucker et al. 2005; Tucker 2007).

Suzuki et al. (2004) compare the *Mus* subgenus to *Apodemus*, the only Eurasian murine taxon with comparable species diversity and geographic range, and conclude that *Mus* are far more ecologically differentiated that *Apodemus*. They also suggest that this ability to rapidly and adaptably change habitats explains the unusually high regional diversity within *Mus,* where species are often sympatric.

In contrast to the diversity of habitats that *Mus* species occupy, the *Mus* subgenus is distinguished by striking chromosomal uniformity and morphological conservatism across species (Auffray & Britton-Davidian 2012). With the exception of some populations of *M. m. domesticus* and the Indian pygmy mouse, *M. terricolor*, all members have a 2n = 40 acrocentric chromosome karyotype (Auffray & Britton-Davidian 2012). In contrast, the other three *Mus* subgenera are karyotypically diverse, including the bizarre sex-reversed XY females in the subgenus *Nannomys* (Veyrunes et al. 2010).

Many species of *Mus* were only discovered recently. For example, six of the 14 currently known species in the *Mus* subgenus have been discovered since 2003, including *Mus cypriacus*, found only on the island of Cyprus, and apparently sister species to *M. macedonicus* (Cucchi et al. 2006). The rate at which new species are being discovered suggests that the full diversity of the genus *Mus* remains to be uncovered (Auffray & Britton-Davidian 2012). In the next few paragraphs, I will summarize some of the biology of the Palearctic *Mus* species (Fig. 1.3), concluding with a more detailed discussion of the mound-building mouse (*M. spicilegus*).

Figure 1.3 Adapted from the IUCN Red List (Cucchi et al. 2006; Amori et al. 2008; Coroiu et al. 2008; Kryštufek & Vohralík 2012) and (Mitsainas et al. 2009). *Mus musculus* is found throughout the area depicted, with the dotted blue line representing the hybrid zone between *M. m. domesticus* in the west and *M. m. musculus* in the east (Kawakami & Butlin 2001). The *Mus spicilegus* range is in yellow, overlapping slightly with the *Mus macedonicus* range in pink. *Mus cypriacus*, only on the island of Cyprus, in purple. The *Mus spretus* range is shown in green.

Fossil evidence of *Mus* in Europe is lacking during the last glacial maximum, suggesting that species currently present in the Palearctic clade originated elsewhere and spread rapidly into Europe in the last 12, 000 years (Thaler 1986). One of the most intriguing Pleistocene fossils from Europe is the giant mouse of Crete, aptly named *Mus minotaurus* (Mayhew 1977). Fossil evidence from archaeological sites (Cucchi et al. 2005), coupled with recent molecular analyses (Rajabi-Maham et al. 2008; Duvaux et al. 2011), suggest that the western house mouse *Mus musculus domesticus* first arrived in the fertile crescent, near Iran, at the beginning of the Neolithic, around the time agriculture began about 12, 000 years ago.

House mouse (*Mus musculus,* Linnaeus, 1758) taxonomy is rather fraught, involving many different classifications that split the house mouse into a species complex, or lumped all the subspecies into a single house mouse species. The current consensus is for *M. musculus* to be a single species, with five subspecies and lab strains that are mosaics of different subspecies or even *Mus* species (Tucker 2007; Auffray & Britton-Davidian 2012). In addition to its utility as a lab model, the house mouse is a speciation model because of a famous hybrid zone between *M. m. domesticus* in Western Europe and *M. m. musculus* in Eastern Europe (Fig. 1.3), and a less famous one between *M. m. domesticus* and *M. spretus*. Possibly because of its rapid radiation, the phylogeography of *M. musculus* remains unresolved, and requires additional specimens from west-central Asia to provide the geographic origin, mode and tempo of the radiation (Suzuki & Aplin 2012).

Mus spretus, Latase, 1883, the Algerian or Western Mediterranean mouse lives in relatively arid grasslands, shrubs and agricultural fields and probably evolved in North Africa (Amori et al. 2008). Its relationship to the other members of the Palearctic clade remains ambiguous

(Fig. 1.2), as nuclear genes place the species as sister to the *M. spicilegus*/*M. macedonicus* clade, whereas mitochondrial genes place *M. spretus* as an outgroup to the rest of the Palearctic taxa (Tucker et al. 2005). This discordance between nuclear and mitochondrial genomic histories could imply historical introgression of nuclear genes between *M. spretus* and *M. spicilegus*/*M. macedonicus*. However, *M. spretus* does not currently overlap in range with either of the other two species. Tucker et al. (2005) also suggest that rate heterogeneity in the loci used could account for ambiguous results in the phylogeny of the Palearctic *Mus*. It hybridizes successfully with the western house mouse subspecies, *M. m. domesticus*, and has become a relatively important genetics model. Like *M. spicilegus*, *M. spretus* has a minute Y-chromosome and a socially monogamous mating system (Cassaing & Isaac 2007; Cassaing et al. 2010; Auffray & Britton-Davidian 2012).

Mus macedonicus, Petrov & Ruzic, 1983, the Macedonian or Balkan short-tailed mouse is found in a wide range of habitats from farmland to scrubland (Kryštufek & Vohralík 2012). A recent study based on nuclear and mitochondrial DNA found a distinct clade in Israel, now a separate subspecies, *M. macedonicus spretoides*. One explanation for why this species has not spread further north, is competitive exclusion with *M. spicilegus* (Orth et al. 2002). The recently discovered Cyprus mouse, *Mus cypriacus* is sister to *M. macedonicus* (Cucchi et al. 2006), with *M. spicilegus* as the next most closely related species (Lundrigan et al. 2002). Intriguingly, reciprocal crosses between *M. spicilegus* and *M. macedonicus* do not result in viable offspring, but both species hybridize successfully with *M. musculus* in the lab (Bonhomme et al. 1978; Sokolov et al. 1983).

Mus spicilegus, Petenyi, 1882, the mound-building mouse

Taxonomy and morphology

The mound-building or steppe mouse was first described as *Mus hortulanus* by Nordmann in 1840, but this name is no longer valid, as the type specimen from a garden in Odessa was probably a house mouse (*Mus musculus*) (Marshall 1986; Macholán 1999). *Mus spicilegus* Petenyi, 1882, with an unspecified type locality near Budapest in Hungary is now the accepted Latin name for the mound-building mouse (Sokolov et al. 1998). Appropriately the word *spicilegus* means "gathering together spikes of grain" (Marshall 1986), and this is the only species in the genus known to build mounds (Auffray & Britton-Davidian 2012). In Hungarian, the species is called *güzüegér*, which translates roughly into "hardworking mouse".

Using the presence of mounds, a new subspecies with a disjoint distribution along the Adriatic coast was discovered. *M. spicilegus adriaticus* is both morphologically and genetically distinct from the main population of *M. spicilegus* found in the north, and is especially distinguished by having exceedingly long and scaly hind feet (Macholan 1996; Krystufek & Macholán 1998; Macholán 2006; Macholán et al. 2007). Even more recently, (Mitsainas et al. 2009) reported a new and divergent mitochondrial lineage of mound-building mice in eastern Greece (Fig. 1.4).

Across the north of its range, *M. spicilegus* is gray-brown, and the same color as the sympatric *M. m. musculus* (Krystufek & Macholán 1998; Sokolov et al. 1998). In contrast, *M. spicilegus adriaticus* in the south is yellowish-brown with a pale belly, resembling the sympatric *M. macedonicus*, but unlike the much darker sympatric *M. m. domesticus* (Krystufek & Macholán

1998). Both subspecies of *M. spicilegus* also have significantly shorter tails than either *M. musculus* subspecies (Krystufek & Macholán 1998).

Figure 1.4 Current *Mus spicilegus* distribution. The distribution in most of Ukraine is doubtful, and in gray. In teal is the main species distribution, in blue is the distinct Adriatic subspecies, and in pink is the recently discovered Sterea Ellada population in eastern Greece. Adapted from (Bauer et al. 1998; Macholán 1999; Coroiu et al. 2008; Mitsainas et al. 2009).

Distribution

Fossils from the Holocene (beginning 12, 000 years ago) are the earliest known records of *M. spicilegus* in Europe (Krystufek & Macholán 1998). At present, the mound-building mouse is distributed as far west as Austria, and extends into northern Slovakia (Bauer et al. 1998) and east into Ukraine (Zagorodnyuk & Berezovsky 1994). For a long time, Bulgaria was thought to be the southern edge of its range, however the recently classified subspecies *M.*

spicilegus adriaticus now occupies a geographically disjoint distribution along the Adriatic coast, at least 250km from the nearest known Serbian populations in the north (Krystufek & Macholán 1998; Macholán et al. 2007). A new Sterea Ellada population exists at low densities in eastern Greece (Mitsainas et al. 2009). *M. spicilegus* is sympatric with the commensal house mouse *M. musculus* throughout its range (Auffray & Britton-Davidian 2012), and with *M. macedonicus* in the south of its range (Fig 1.3).

Evidence for reproductive barriers is mixed. Behavioral trials show that although *M. macedonicus* females and *M. musculus* of both sexes are eager to approach *M. spicilegus* of the opposite sex, the mound-building mice give these heterospecific suitors a chilly and often aggressive welcome (Ivantcheva & Cassaing 1999). However while *M. m. musculus* males spent more time investigating the urine of conspecific females in estrous, male mound-building mice did not appear to discriminate between conspecific and heterospecific females (Kotenkova et al. 1989). More convincing evidence of pre-copulatory isolating mechanisms come from a study that reared *M. spicilegus* and *M. musculus* in mixed litters, and still found that individuals of both sexes from both species spent longer investigating the ano-genital odor of their own species, regardless of familiarity (Heth et al. 2003).

Habitat

Unlike the house mouse, *M. spicilegus* avoids forests and human settlements, and prefers a variety of open habitats, like the other non-commensal Palearctic *Mus* species (Coroiu et al. 2008). In particular, the mound-building mouse is found in natural steppe grasslands and agricultural fields. In Hungary at least, this species appears to prefer agricultural areas to natural grasslands, possibly because they prefer the soil type used for agriculture (Bihari

2004). The current IUCN conservation status of *M. spicilegus* is listed as of least concern (Coroiu et al. 2008). However the population is decreasing, and the loss of steppe grasslands and agricultural intensification constitute major threats that may cause further declines (Macholán 1999). The species is highly endangered in Austria, one of the more economically and agriculturally developed parts of its range (Hölzl et al. 2011a), but considered an agricultural pest in Hungary (Bihari 2004).

Mounds

In autumn, from mid-August to late September or even November, *M. spicilegus* build mounds of soil and vegetation that range from 0.5 to 4m in diameter (Sokolov et al. 1998). Occasionally, mice will construct mounds as early as June or July, when the cereal crops grown in fields are ripening, and the mice are still breeding. In these cases, reported from the Odessa region of Ukraine, the mounds are almost invariably destroyed by ploughs shortly after harvest, but can be rebuilt in as few as 3-4 days (Muntyanu 1990). In most cases, mounds are built on field margins, weedy fields, or cultivated land, and construction usually takes 14-21 days (Sokolov et al. 1998). Mound densities have been reported from 7-20 mounds per hectare in crop fields, to as few as one mound per hectare on field margins (Muntyanu 1990) (Table 1.1). When mounds are not destroyed, they may be re-used the subsequent year (Sokolov et al. 1998, personal observations).

Location	Country	Mound Length (m)	Mound height (m)	No. of mounds	Mice per mound	Capture method	Quantity of plant matter in mound	Mound density (mounds/ha)	Nest depth (m)	Reference	Notes
Dnepropetrovsk	Ukraine	4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Sokolov 1998	Sandy soil
Kechnec	Slovakia	2.22±0.3	0.29±0.8	175	4-21	Live traps around mounds for 2 days	10-50 L	0.7-10	0.2-0.45	Canady 2009	
4 locations	Austria/ Slovakia	Max. 3	Max. 0.38	109	1-11	Live traps around mounds	N/A	10-24	N/A	Holzl 2009	Only give mound volumes 165.51±13.51 cm^3 2.11-717.81cm^3
10 locations	Hungary	2.47±0.23	0.27±0.04	93	6.6±2.1	Digging with a trench	38.0±10.1 L	N/A	0.6±0.25	Szenczi 2011	Compared clay to sand, only differences are sand had nests twice as deep and more plants in mounds
Gyongyos	Hungary	N/A	N/A	10	1-23	Digging with a metal barrier	N/A	N/A	N/A	Poteaux 2008	Probably the same mice as Gouat 2003
Many locations	Hungary	2.2	0.26	242	4-8	N/A	Max. 10kg	Max. 25	0.15-0.4	Bihari 2004	
Odessa	Ukraine	2	0.3-0.6	>200	5-14	N/A	3-5 kg	1-20	0.3-2.2	Muntyanu 1990	Known as Moldavia in the paper
Calarasi	Romania	2.81	0.37	52	N/A	N/A	N/A	N/A	N/A	This report	
Srebarna	Bulgaria	2.36	0.35	30	N/A	N/A	N/A	N/A	N/A	This report	
Krushovitsa	Bulgaria	2.94	0.34	15	N/A	N/A	N/A	N/A	N/A	This report	
Pleven	Bulgaria	N/A	N/A	40	2-11	Digging	N/A	N/A	N/A	Garza 1997	
Telish	Bulgaria	2.49	0.21	93	N/A	N/A	N/A	N/A	N/A	This report	
Telish	Bulgaria	2.84	0.41	17	N/A	N/A	N/A	N/A	N/A	This report	
Rakita	Bulgaria	2.46	0.34	8	N/A	N/A	N/A	N/A	N/A	This report	
Sterea Ellada	Greece	Most <1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Mitsainas 2009	One record of a nest in

Mound building, a behavior unique to this species within the genus *Mus*, appears to be flexible. Captive groups in a Bulgarian laboratory made nests, but not mounds (Simeonovska-Nikolova & Mehmed 2009), and there is a single report of mice from the Sterea Ellada population in eastern Greece living in a nest in a discarded plastic irrigation tube in winter (Mitsainas et al. 2009). In addition, mounds from this population are generally smaller than 1m, in contrast to mounds in the northern part of the species range, which range from 1-4m (Table 1.1) (Muntyanu 1990; Sokolov et al. 1998; Canady et al. 2009; Hölzl et al. 2009; Szenczi et al. 2011). A more intensive sampling of this Sterea Ellada population and across the range of the southern subspecies, *M. spicilegus adriaticus* would help reveal the extent to which mound-building is correlated with climate.

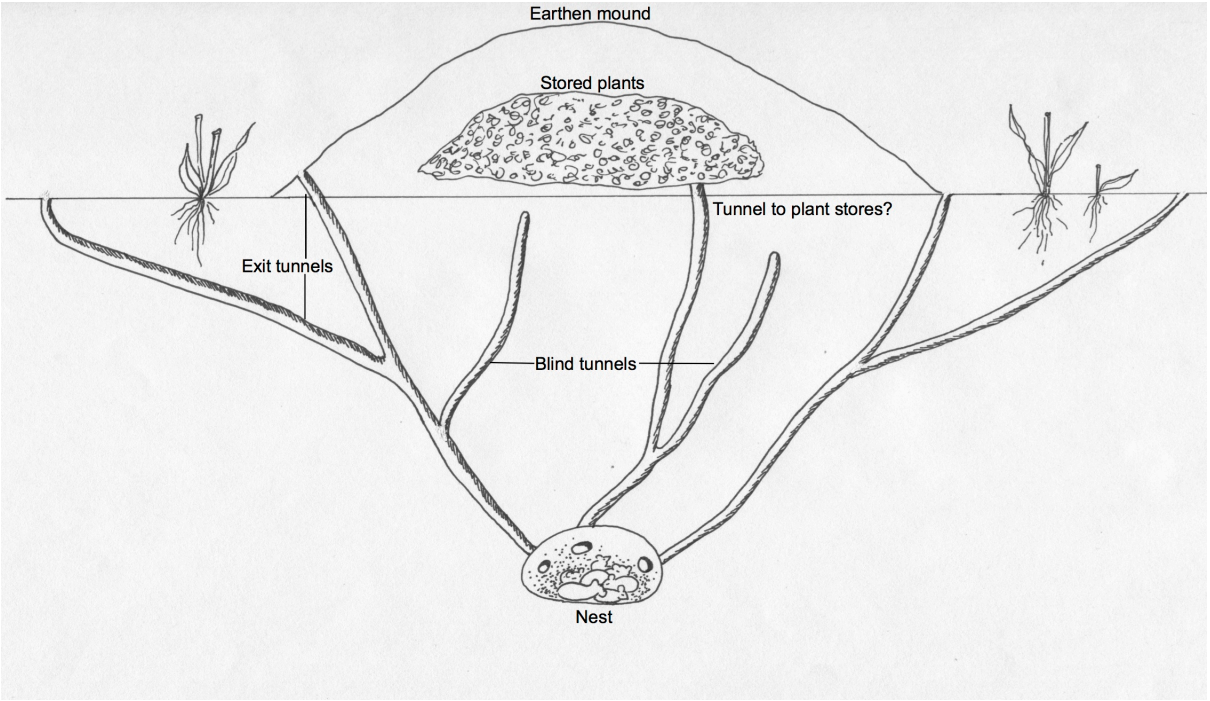


Figure 1.5 Diagram of a mound composed of vegetation and soil that is above ground, with a network of tunnels leading to a nest chamber underground below the mound.

Mounds have a relatively stereotyped structure (Fig. 1.5) and construction sequence. Mice will first pile up 10-50L of vegetation collected from the surrounding area, and proceed to

cover the plants with soil. While still covering the mounds with soil, the mice will begin digging tunnels under the mound (Hölzl et al. 2009). When complete, a mound (including both plants and soil) is typically oval or cone-shaped, 140-170L in volume, but can be as large as 700L (Hölzl et al. 2009; Szenczi et al. 2011), with a network of tunnels underneath, and at least one nest chamber lined with grass. The depth of the nest chamber appears to vary with soil type, with nests in sandy soil that are twice as deep underground compared to nests in clay (Szenczi et al. 2011). The number of holes leading to a mound can vary from a few to almost 50, and correlates positively with mound volume (Hölzl et al. 2009). These holes can be as far as 2.5m from the edge of the mound, and additional tunnels can end just shy of the surface (Muntyanu 1990). Within a particular geographic region, mound size probably increases with the number of mice inside the mound (Sokolov et al. 1998; Szenczi et al. 2011) but see (Hölzl et al. 2009). Mound volume also seems to increase with the availability of suitable plant construction materials within 3m of the mound (Hölzl et al. 2009), and larger mounds are filled with more plants (Szenczi et al. 2011).

Many questions about mounds remain unanswered, particularly with respect to their function. The vegetation used in a mound varies with location, as does the area over which mice forage for building materials, which can range from 10 to 140m^2 (Muntyanu 1990; Sokolov et al. 1998). At least 84 species from 29 plant families have been found in mounds (Sokolov et al. 1998), including species from the genera *Echinochloa* (Hungary), *Ambrosia* (Hungary), *Matricaria* (Hungary), *Amaranthus* (Austria), *Setaria* (Austria, Hungary, Slovakia), *Chenopodium* (Austria, Hungary, Slovakia), *Solidago gigantea* (Slovakia), and much less frequently (5%) sunflower seeds and wheat ears (Festetics 1961; Bihari 2004; Canady et al. 2009; Hölzl et al. 2009; Szenczi et al. 2011).

An obvious conclusion to draw is that mounds contain food stores for the overwintering mice beneath. Although this hypothesis is widely asserted (Muntyanu 1990; Sokolov et al. 1998), a recent study in Hungary found almost no overlap between the plants in mounds and those found in mouse feces (Szenczi et al. 2011). One possible explanation for the lack of overlap is the spatial and temporal sampling used in this study. While 93 mounds were examined across ten locations from October to March (a single winter) for the composition of plant material, only 21 mounds from two of those locations were used for fecal sampling, and this took place in March, towards the end of winter, when the mice may well have eaten most of the more palatable food in the mounds. Another study looking at 242 mounds across Hungary found cereal crops in less than 5% of the mounds, even though the mounds were constructed in agricultural fields (Bihari 2004). In the laboratory, wild-caught mice did eat seeds from all three of the most common plant species found in nearby mounds, but appeared to prefer to eat *Setaria* seeds, in spite of building preferentially with *Amaranthus* and *Chenopodium* (Hölzl et al. 2011b). In addition, at least two studies reported no tunnels connecting the plant caches to nests underneath (Canady et al. 2009; Szenczi et al. 2011). Evidence for mounds serving as food stores is decidedly mixed.

A second, non-mutually exclusive and distinctly attractive hypothesis for the vegetation in mounds is that it provides insulation, and even heat by fermenting gently all winter (Hölzl et al. 2011a), in the manner of Australian megapode mounds that incubate eggs (Frith 1956). Mounds do indeed provide substantial insulation against the cold, with temperatures under mounds being warmer than soil at the same depth outside mounds (Szenczi et al. 2011), and a positive correlation between mound size and thermal stability (Hölzl et al. 2011a).

Temperature and pH measurements confirm that no fermentation occurs (Hölzl et al. 2011a). Mounds also appear to keep the soil underneath drier, and larger mounds are more effective at keeping moisture out than smaller mounds (Szenczi et al. 2011). Certainly survival within mounds varies, possibly with temperature, with almost 100% survival in a Hungarian study where the surface soil temperature ranged from -3ºC to 3ºC, and mean nest temperatures were 8ºC, about 2ºC warmer than temperatures at the same depth outside mounds (Szenczi et al. 2011). In contrast, almost half the mice were dead in excavated mounds in the Ukraine, where temperatures ranged from -5ºC to -10ºC, and only 0ºC in nest chambers. The surviving mice were in torpor, and would come out of torpor after a few hours at room temperature (Muntyanu 1990). Intriguingly, *M. spicilegus* winter fur is less insulating than the winter fur of *M. m. musculus* (Muntyanu 1990), consistent with the notion that mounds help to offset the need for insulating fur. Huddling amongst *M. spicilegus* has yet to be studied.

Reducing predation risk is the most speculative hypothesis for mound function (Bihari 2004). Anecdotal accounts suggest that ground predators seldom excavate mounds (Szenczi et al. 2011). Nocturnal predators must be a threat, as weasels (*Mustela nivalis*) have been trapped in the vicinity of mounds (Canady et al. 2009, personal observations), and video recordings show that mice only venture above ground to forage and build mounds at night, or early dawn (Hölzl et al. 2011b). The degree to which mounds protect (or possibly attract) predators deserves further investigation.

In addition to the mystery of mound function, records of who actually builds the mounds are surprisingly patchy and inconsistent. One of the most thorough treatments suggests that

only a subset of the mice in a mound actually contribute to its construction (Festetics 1961). Some authors state that juveniles 3-4 weeks old build mounds (Sokolov et al. 1998), whereas others claim that parents build mounds and stock them with grain (Garza et al. 1997). One study begins with the assumption that only juveniles (42 days old) build mounds, and used cotton wool as a substitute for soil and plants (Serra et al. 2012). To the extent that moving six cotton balls a day constitutes mound building, this experiment revealed that only two out of six mice transported almost 80% of the cotton balls across a total of four days. Interestingly, there was no observable sex difference, with a total of five female and seven male carriers. As the mice took about 15min to complete their daily task, it would be intriguing to repeat a similar experiment with more building material and more individuals of different ages.

Sociality

Regardless of which mice build the mounds, the sheer volume of a mound, and the area over which mice must forage for building materials makes mound-building a costly activity that benefits all those who live under the mound equally. The next question is who actually lives under the mounds in winter? The exact number of mice reported varies with capture method (Table 1.1), but appears to range from 1-23 individuals in a mound, almost all of which are juveniles, with the exception of the occasional adult (Muntyanu 1990; Garza et al. 1997; Poteaux et al. 2008; Canady et al. 2009; Hölzl et al. 2009; Szenczi et al. 2011). Based on microsatellite genotyping, each mound can contain the offspring of up to 2 males and 2 females, and mice from the same mound are more related to each other than mice from different mounds (Garza et al. 1997; Poteaux et al. 2008). Presumably kin gain greater inclusive fitness benefits from huddling in a communal mound. Intriguingly, mound-building

mice appear to move between mounds, although the timing and frequency of this movement is unpublished, and other free-riding species, including *Apodemus agrarius*, are occasionally found within mounds (Hölzl et al. 2009).

Odor seems to provide a means of kin recognition in *M. spicilegus*. Captive male mound-building mice from the same wild population were able to distinguish male kin from non-kin by odor alone, and could even make finer-scale discriminations between cousins (r=0.125) and double-cousins (r=0.25) (Busquet & Baudoin 2005). Similarly, male mound-building mice from source populations over 100km apart were able to distinguish more- and less-genetically related males based on odor alone, and in spite of sharing a strong-smelling aniseed diet with the less-related males (Colombelli-Negrel & Gouat 2006; Raynaud et al. 2012). Females are also able to distinguish unfamiliar sisters from non-kin by smell alone (Baudoin et al. 2005).

Consistent with its habit of overwintering in groups comprising more than one litter, *M. spicilegus* appear unusually tolerant of familiar conspecifics. In the wild, there is no evidence of aggression between mice from the same mound (Sokolov et al. 1988). In the laboratory, adults of both sexes are more sociable towards same-sex individuals with which they were raised than towards unfamiliar individuals, but as juveniles, these mice are equally sociable towards familiar and unfamiliar juveniles. By adulthood, males still show almost no aggression towards males with which they had been raised. In contrast, male house mice are equally aggressive towards familiar and unfamiliar males, but less aggressive than unfamiliar adult male *M. spicilegus* are to each other. Similarly, adult female *M. spicilegus* are only aggressive towards unfamiliar females, and more so than unfamiliar female *M. musculus* are to

each other, while both species show no aggression towards familiar females (Szenczi et al. 2012), consistent with female kin-group formation and communal breeding in *M. musculus*, and with group living within mounds in *M. spicilegus*. As adults, mound-building mice of both sexes display markedly high levels of aggression towards unfamiliar individuals (Suchomelova et al. 1998; Patris et al. 2002; Simeonovska-Nikolova 2003, 2008).

Interestingly, aggression and unfamiliarity may be necessary for reproduction. Consistent with their life history of overwintering in groups of potentially unrelated juveniles (Garza et al. 1997), pairs that are reared together as juveniles almost always fail to reproduce. However, being re-paired with an unfamiliar individual triggers reproduction in spite of the greater aggression displayed by both males and females towards a new mate (Busquet et al. 2009).

In addition to their greater aggression towards unfamiliar conspecifics, mound-building mice differ strikingly from commensal house mice by displaying almost no neophobia, proceeding to engage almost instantly and repeatedly with a new object, often with destructive consequences (Meshkova et al. 1985). Putting mice in enclosures 4x4x2.5m showed that house mice explore a new space vertically, whereas the non-commensal *M. spretus*, *M. macedonicus* (*M. abbotti* in this paper) and *M. spicilegus* tended to remain on the ground (Meshkova et al. 1999; Simeonovska-Nikolova 2000).

The sexes differ in their responses to new environments. In a novel attempt to quantify the strength of social bonds, Bardet and colleagues (2007) constructed a challenge for the mice that involved swimming across a water obstacle to reach a group of conspecifics. The notion was that the company of familiar mice would be more desirable, and so individuals would

hesitate less before braving the water to join their companions. The experiment was run in two stages: one without water, so that mice simply had to move across a corridor to join other mice, and a second stage where the corridor was filled with water, and constituted more of a challenge. In the absence of water, males scurried over almost immediately to join their companions, regardless of familiarity, whereas females only crossed over quickly to join familiar individuals or unoccupied space. Faced with unfamiliar females on the other side, females took significantly longer to join the group. In contrast, when water had been added, none of the females was eager to cross, whereas males that had no mice or familiar males waiting on the other side swam over almost immediately, but males faced with unfamiliar males took significantly longer to cross (Bardet et al. 2007).

Life history and dispersal

Unlike the other Palearctic *Mus*, *M. spicilegus* breed seasonally from late March to mid-October in the northern part of their range (Sokolov et al. 1998). In spring, most mice disperse from mounds to breed, although some females remain within mounds for their first litter, and lactating females are the last to leave the mounds (Muntyanu 1990; Simeonovska-Nikolova & Gerasimov 2000). In early spring, all the pregnant females are 6-8month old adults that overwintered in the mounds, whereas by summer, a second cohort of young females will be old enough (3mths) to reproduce. Each female can have 4-5 litters in a breeding season, with litter sizes ranging from 4 to 11 (Sokolov et al. 1998). Mound-building mice have never been observed to breed during the winter, and few adults have been reported within mounds in the middle of winter, so presumably most adults die before the next spring (Muntyanu 1990; Canady et al. 2009). The breeding habits of the southern subspecies, *M. spicilegus adriaticus* have yet to be documented.

During the breeding season, adults have a home range size of 150-260 m^2 (Sokolov et al. 1998). Slightly over half the females in a field in Hungary had ranges that overlapped with those of other females, but no males were trapped in the same area (Gouat et al. 2003). However, more than three times as many females as males were trapped in this population, which could explain the spatial associations suggestive of polygyny. Females caught in the same trap were significantly more related than a random pair of females, and females with overlapping ranges had the same average relatedness as individuals from the same mound in winter (Poteaux et al. 2008). In contrast, males and females trapped in the same area were less related than males and females from the same mound (Poteaux et al. 2008). Even with a relatively even sex ratio in a Bulgarian population during the summer, male ranges would sometimes overlap with those of more than one female, although most adult mice were found in spatially-associated male-female pairs (Simeonovska-Nikolova 2007). Sex ratios in the wild vary across time and space, with no discernable pattern (Table 1.2).

Table 1.2. Table of different sex ratios caught in different studies, using different methods.

Location	Country	Capture method	Month	Year	Number of males	Number of females	Reference
Kechnec	Slovakia	Live traps around mounds for 2 days	Oct-Mar	2004/ 2005	101	80	Canady 2009
Kechnec	Slovakia	Live traps around mounds for 2 days	Oct-Mar	2005/ 2006	50	33	Canady 2009
Pleven	Bulgaria	Digging	Nov	1992	86	81	Garza 1997
Gyongyos	Hungary	Digging with a metal barrier	Oct	1999	51	32	Poteaux 2008
Gyongyos	Hungary	Trapping grid	April	2000	10	33	Gouat 2003
Pleven	Bulgaria	Trapping grid	May	1994	33	28	Belcheva 2001
Pleven	Bulgaria	Trapping grid	July	1994	58	41	Belcheva 2001
Pleven	Bulgaria	Trapping grid	Sep	1994	12	28	Belcheva 2001
Pleven	Bulgaria	Trapping grid	Nov	1994	10	4	Belcheva 2001
Pleven	Bulgaria	Trapping grid	April-May	1992	13	6	Simeonovska- Nikolova 2007
Pleven	Bulgaria	Trapping grid	June-July	1992	57	42	Simeonovska- Nikolova 2007
Pleven	Bulgaria	Trapping grid	Sep	1992	9	11	Simeonovska- Nikolova 2007
Pleven	Bulgaria	Trapping grid	Nov	1991	9	3	Simeonovska- Nikolova 2000

Consistent with the spatial organization of *M. spicilegus* during the breeding season, experiments involving 2-3 pairs of unfamiliar mice in two adjoining rooms each 2.9x2.3x2.2m showed that while mound-building mice formed male-female pairs with discernable territories, female house mice tended to form spatial groups of kin (Dobson & Baudoin 2002). Even when a similar experiment was repeated that gave females the opportunity to live with sisters, mound-building mice formed stable, spatially distinct pairs with no female kin cohabitation (Baudoin et al. 2005).

Mating system

The mound-building mouse is the first *Mus* to be described as monogamous (Patris & Baudoin 1998; Auffray & Britton-Davidian 2012). The first evidence of social monogamy was female choice, whereby both estrous and postpartum estrous females preferred to copulate with their familiar mate than with an unfamiliar male. In contrast, polygynous house mouse females in estrous actively preferred to mate with unfamiliar males (Patris & Baudoin 1998). When reproductively active *M. spicilegus* are placed in a group in artificially close proximity of 1x1x1m containers, individuals are highly aggressive and establish a dominance hierarchy resulting in only the most dominant pair breeding (Simeonovska-Nikolova 2003).

Indeed, female choice appears to be important in mound-building mice. Females that remain with their preferred male (in two choice tests) display increased neurogenesis in the olfactory bulb relative to females paired with a less-preferred male (Baudoin et al. 2005). In the absence of previous social interaction, females seem to agree with each other on the most attractive male, although the precise criteria of attractiveness remain elusive (Beigneux et al.

2012). In an experiment that did not give mice a choice before pairing them with unrelated mates, more than 50% of the couples failed to reproduce (Busquet et al. 2009), another suggestion that mate choice is important in *M. spicilegus*.

Other aspects of female behavior appear consistent with social monogamy, or, at least, with a female preference for not sharing a mate. Mound-building females are significantly more aggressive towards unfamiliar conspecifics than unfamiliar *M. musculus* females are to each other (Sokolov et al. 1988; Suchomelova et al. 1998; Patris et al. 2002; Szenczi et al. 2012). In addition, being housed in female groups suppresses sexual receptivity in female mound-building mice (Féron & Gheusi 2003). An experiment showed that two sisters sharing a mate each produced fewer offspring per litter, and had longer inter-litter intervals than females with a male to themselves. Indeed by the end of this experiment, males in trios did not have more offspring than males in pairs. As no agonistic interactions were recorded between the females in trios, the authors conclude that some other mechanism like pheromone production suppressed reproduction in the polygynously mated females (Gouat & Féron 2005).

Paternal care is one of the much-cited pieces of evidence in favor of social monogamy in mound-building mice. It also provides another compelling reason for females to be choosy and to prefer monogamy to polygyny. Compared to house mice, male *M. spicilegus* spend significantly longer covering their week-old pups, are more efficient at retrieving straying pups, and alternate pup protection with females (Patris & Baudoin 2000). Furthermore, the more time a male spends in the nest with his mate, the shorter the intervals between her litters, suggesting that paternal care does translate into significant reproductive benefits

(Féron & Gouat 2007), presumably by reducing the energetic costs that a female has to invest in reproduction.

In contrast to the behavioral evidence for social monogamy, evidence of intense sperm competition in this species suggest that the mound-building mouse is far from genetically monogamous. In particular, *M. spicilegus* have the largest testes for their body mass of the Palearctic *Mus* species (Gomendio et al. 2006; Frynta et al. 2009), in absolute terms, their testes are two to three times as heavy as those of the larger and polygynous *M. m. musculus* (Sokolov et al. 1998). As relative testis size is a strong correlate of sperm competition across taxa as diverse as butterflies (Gage 1994), fish (Stockley et al. 1997), birds (Moller 1991) and mammals (Gomendio et al. 1998), many papers simply use it as a proxy for sperm competition. Compared to other *Mus*, *M. spicilegus* also have the highest rates of sperm capacitation – presumably the faster to fertilize ova, and the greatest proportion of capacitated sperm that undergo the acrosome reaction in response to progesterone, an in vitro assay meant to simulate sperm contacting an egg. Among the four *Mus* species studied, both these sperm traits were positively correlated with relative testis size (Gomendio et al. 2006). In a phylogenetically controlled study of 18 muroid rodents that included *M. spicilegus*, the total number of sperm, and measures of sperm quality including the percentage of normal sperm, motile sperm and sperm with intact acrosomes were all positively correlated with relative testis size (Gómez Montoto et al. 2011a). Similarly, sperm swimming speed and sperm head size are both positively correlated with relative testis size in 11 muroid rodents including *M. spicilegus*, and that sperm head area was largely dependent on the length of the sperm head and the presence of an apical hook (Fig. 1.6) (Gómez Montoto et al. 2011b).

Confusingly, sperm head size is reputedly smaller (mean 7.57 by 3.07 μm) than other species in the *Mus* subgenus (Sokolov et al. 1998).

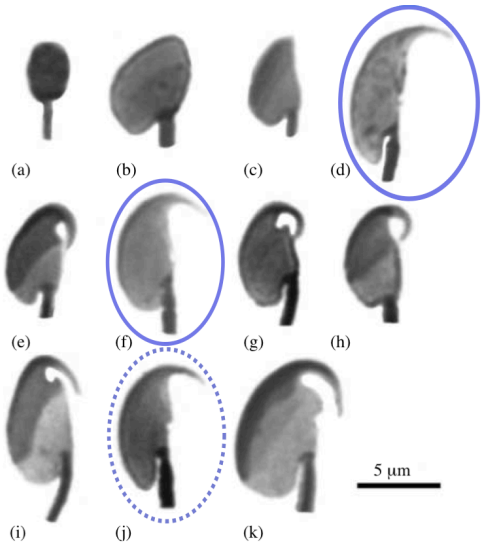


Figure 1.6 Sperm head morphology in 11 muroid species, with the three *Mus* species outlined, and M. spicilegus in the dotted circle. (a) *Microtus duodecimcostatus*, (b) *Microtus cabrerae*, (c) *Microtus lusitanicus*, (d) ***Mus musculus***, (e) *Microtus arvalis*, (f) ***Mus spretus***, (g) *Arvicola sapidus*, (h) *Clethrionomys glareolus*, (i) *Chionomys nivalis*, (j) ***Mus spicilegus*** and (k) *Apodemus sylvaticus*. Modified from (Gómez Montoto et al. 2011b).

Although an apical hook is known to aid in forming sperm trains that swim faster than individual sperm in murids like *Apodemus sylvaticus* (Moore et al. 2002), which has the largest, most hooked sperm head in Figure 1.6, there is no evidence that *Mus* sperm regularly form trains or clumps; nor is there an intra-specific correlation between sperm competition and hook curvature in house mice (Firman & Simmons 2009). Further confusing the matter, the length of the sperm midpiece is the only known predictor of sperm velocity in *M. musculus* (Firman & Simmons 2010) suggesting that a more detailed treatment of how sperm form and function within the *Mus* subgenus could yield different conclusions from a study across muroids.

CHAPTER 2

Phylogeography and population genetics of mound-building mice (*Mus spicilegus*)

Summary

Mound-building mice (*Mus spicilegus*) are closely related to the widespread human commensal, house mice (*M. musculus*), but differ from other *Mus* species in their construction capabilities. The habit of gathering to build and overwinter under large earthen mounds instead of capitalizing on barns and houses makes *M. spicilegus* an interesting candidate for examining the influence of ecology and social behavior on phylogeography and population structure. This study uses mitochondrial control region sequences and microsatellites to investigate phylogeographic relationships and population genetic structure across the entire species range, and at a finer scale of 5-200km. Evidence from both markers suggests low levels of geographic structure compared to *M. musculus*, and raises the possibility that a geographically isolated subspecies, *M. s. adriaticus*, is less genetically distinct from northern *M. spicilegus* populations than was previously thought. One interpretation of the relative lack of structure is a recent population expansion into the current species range, another is that mound-building mice disperse over relatively large distances compared to other *Mus*.

Introduction

The genus *Mus* is particularly interesting because although the house mouse is a workhorse of genetics and neurobiology, its evolutionary history is still being unraveled (Duvaux et al. 2011). In particular, the western house mouse, *Mus musculus domesticus* appears to have expanded into Europe in the Neolithic, as recently as 12, 000 BC (Rajabi-Maham et al. 2008), probably in the wake of human agriculture (Cucchi et al. 2005).

In contrast to the house mouse, the other European members of the *Mus* genus are not human commensals, and are likely to have a different phylogeographic history. In particular, the mound-building mouse (*Mus spicilegus*) makes an interesting comparison with the house mouse because instead of relying on humans for food and shelter, it constructs overwintering mounds of soil and vegetation (Muntyanu 1990; Sokolov et al. 1998; Macholán 1999). A mtDNA analysis suggests that *M. spicilegus* diverged from its sister species, *Mus macedonicus* between 700 000 and 1 million years ago (Macholán et al. 2007).

The mound-building mouse is distributed across the steppes of Eastern Europe, but has recently been found in a discontinuous population in the southern Balkans (Krystufek & Macholán 1998). This population is both morphologically and molecularly distinct, and has been classified as a new subspecies, *Mus spicilegus adriaticus* (Macholán et al. 2007). Just adjacent to this Adriatic population is an even more recently discovered population in eastern Greece that is genetically distinct from populations in western Greece (Mitsainas et al. 2009). Interestingly, these southern populations appear to construct smaller mounds than the populations in the northern part of the species range, with a single observation of a mound

less than 0.5m in diameter in Greece, whereas most mounds in the north of the species' range are 1-3m in diameter (Table 1.1).

The phylogeographic history of *M. spicilegus* remains unresolved, possibly because the Pleistocene was a time of dramatic climate change due to repeated glaciations, resulting in a constantly changing environment and complex population histories for many species (Hofreiter & Stewart 2009). Evidence from mtDNA suggests that *M. spicilegus* colonized Europe from the north of the Black Sea and spread south (Macholán et al. 2007). However the recent addition of haplotypes from eastern Greece that appear to be basal in the species tree suggests that mound-building mice crossed into the Balkans through the Bosporus land bridge and spread both north into the Pannonian steppes and south into eastern Greece.

This study investigates the population history and geographic structure of mound-building mice in Europe by using additional samples to the north and south of the species range, as well as by analyzing a complementary microsatellite dataset.

The main questions are:

1. What route did *Mus spicilegus* take in colonizing Europe?

2. How is the recently discovered Sterea Ellada population from Greece related to the rest of the species?

3. Do the significant genetic differences between phenotypically and geographically different populations (currently classified as *M. spicilegus* and *M. spicilegus adriaticus*) persist with a more complete sampling across the species' range?

4. Is there significant population structure within the main range of *M. spicilegus* in the north?

Methods

DNA sample sources

We obtained DNA samples from a total of 24 locations across the known range of *Mus spicilegus*, 13 of which are unique to this study, and were obtained by trapping mice or from museums (Fig. 2.1, Appendix Table 1). Tail tips were preserved in 99% ethanol prior to DNA extraction. The remaining sequences used in the phylogeographic analyses are from GenBank (Table 2.1). In total, 238 sequences of 877bp from the mitochondrial control region were used. Individuals from 16 locations were also to generate a total of 503 multilocus microsatellite genotypes (Fig. 2.1).

Figure 2.1 In green is the current documented range of *Mus spicilegus* adapted from (Mitsainas et al. 2009). There are probably mice in the Ukraine between the eastern most location and the main chunk of green, but none recorded in the last 30 years. Stars represent the sampling locations for the mtDNA sequences, and the circled stars are locations for which there is also microsatellite data.

Table 2.1. Sample sources, mtDNA haplotypes and microsatellite population groupings.

Country	Locality	Latitude ºN	Longitude ºE	mtDNA haplotype (N)	GenBank	Microsatellite group (N)
Austria	Waldacker	47.939214	16.97134	AHS1 (3) AHS2 (1)	This report	
Austria	Halbturn	47.858324	16.97525	AHS1 (1)	U47536	
Austria	Mönchhof	47.88211	16.94231	A1 (2)	EU106300 EU106299	
Hungary	Unknown	Unknown	Unknown	H1 (2) H2 (1) AHS1 (1) AHS2 (2)	This report	3 (15)
Hungary	Kápolna	47.759113	20.24708	AHS1 (25) H3 (3) AHS2 (6)	This report	5 (65)
Hungary	Sződ	47.724664	19.17139	H3 (7) H4 (5) H5 (1) H6 (1) AHS2 (16)	This report	4 (47)
Slovakia	Komárno-Ďulov Dvor	47.78647	18.16795	AHS1 (13)	This report	2 (15)
Slovakia	Vrbová nad Váhom	47.849314	18.05090	AHS1 (3)	EU106306 EU106307 This report	1 (3)
Slovakia	Kechnec	48.549383	22.26444	AHS2 (39) Slo1 (1)	This report	6 (40)
Slovakia	Belža	48.580792	22.27416	AHS2 (3)	This report	7 (3)
Serbia	Debeljača	45.066667	20.6	Se4 (1)	This report	
Serbia	Pančevo	44.855868	20.69824	Se1 (1) Se2 (1) Se3 (1)	EU106308 EU106309 EU106310	
Bulgaria	StrainZBN	Unknown	Unknown	B5 (1)	AB039263	
Bulgaria	Srebarna	44.094442	27.06402		This report	8 (34)
Bulgaria	Knezha	43.497984	24.08117		This report	12 (86)
Bulgaria	Telish	43.327022	24.26103	B1 (3) B2 (3) B3 (2)	This report	11 (224)
Bulgaria	Krushovitsa	43.348975	24.41527	B1 (6) B2 (1)	This report	9 (7)
Bulgaria	Rakita	43.285017	24.23035	B4 (1)	This report	10 (31)
Moldova	Kishinev	47.085085	28.78417	Mol1 (1) Mol2 (1) Mol3 (1)	U47537 EU106321 EU106322	
Ukraine	Dshankoi	45.708611	34.39333	U1 (1)	U47538	
Montenegro	Ulcinj	42.929722	19.22429	Mon1 (1) Mon2 (1) Mon3 (1)	EU106311 EU106312 EU106313 EU106301	
Greece	Igoumenitsa	39.50615	20.26553	G1 (1) G2 (1)	EU106314 EU106315	13 (2)
Greece	Vlaherna	39.172623	20.99896	G5 (1) G4 (1) G3 (1)	EU106316 EU106317	14 (2)
Greece	Komeno	39.046958	22.03213	G3 (1)	EU106302 EU106303	15 (2)
Greece	Patras1	38.249626	22.73545	G6 (1)	EU106318 EU106304 EU106305	16 (5)
Greece	Patras2	38.266469	22.74992	G7 (1) G8 (1)		16
Greece	Rozena	38.119795	22.39723	G9 (1)	EU626226	
Greece	StereaEllada	38.604393	22.71521	G10 (2)	EU626224 EU626225	

DNA extraction, mtDNA sequencing and microsatellite genotyping

We extracted genomic DNA from tail tips using a Qiagen DNeasy Blood and Tissue Kit (Qiagen). 877bp of the mitochondrial control region was amplified using the following two sets of primers: MusPro9F (5'AAGGAGCTACTCCCCACCAC3') and MusCR603R (5'GCCTTGACGGCTATGTTGAT3'), and MusCR459F (5'AAATGCGTTATCGCCCATAC3') and MusPhe984R (5'GCATTTTCAGTGCTTTGCTTT3'). PCR reactions were performed in a 15µl reaction containing 2.5µl of 10x reaction buffer, 0.3µl of 10mM dNTPs, 0.3µl of 25mM $MgCl_2$, 0.2µl of Amplitaq DNA polymerase (Applied Biosystems), 2.5µl of each 10 µM primer and 2.0µl of 5-10ng/µl genomic DNA. We used an Eppendorf Mastercycler ep (Eppendorf) for the reactions with one cycle of 2min at 94ºC, followed by 30 cycles of 15s at 94ºC, 15s at 60ºC and 60s at 72ºC, and a final cycle for 60s at 72ºC. Cleaned PCR products were sequenced with BigDye Terminator Kit 3.1 (Applied Biosystems), Following an ethanol precipitation, we then ran cycle-sequenced products on an ABI automated sequencer ABI3130xl (Applied Biosystems) and edited and aligned the sequences using Geneious 5.6 (Drummond et al. 2012).

In addition, we amplified ten autosomal and four X-linked microsatellites using primers from (Garza et al. 1997; Schalkwyk et al. 1999). PCR reactions were amplified in a 15µl reaction containing 2.5µl of 10x reaction buffer, 0.3µl of 10mM dNTPs, 0.3µl of 50mM $MgSO_4$, 0.15µl of Amplitaq DNA polymerase (Applied Biosystems) and 2.0µl of 30-40ng/µl genomic DNA. To each reaction, we also added 0.54µl of a primer tag labeled with the fluorescent dye 6-FAM (Applied Biosystems) that would bind to 0.06µl of the forward primer tagged on the 5' end with a complementary CAG sequence

(CAGTCGGGCGTCATCA), and 0.6µl of the reverse primer. All three primers were at the same concentration of 10µM. We used an Eppendorf Mastercycler ep (Eppendorf) for the reactions with one cycle of 3min at 94ºC, followed by 40 cycles of 30s at 94ºC, 45s at an optimized annealing temperature (Table 2.2) and 60s at 72ºC, and a final cycle for 10min at 72ºC. To visualize the microsatellites, 3µl of each PCR product was then combined with 0.3µl of the internal size standard ROX 400HD (Applied Biosystems) and 20µl of formamide, and run in an ABI 3730xl Genetic Analyzer (Applied Biosystems). To minimize errors, samples that were ambiguous were re-scored from new PCR reactions. Individuals genotyped for fewer than 8 loci were excluded from the analyses. Genotypes were scored using Peak Scanner 2.0 (Applied Biosystems).

Table 2.2 Primer sequences and optimized PCR annealing temperatures for 14 microsatellite loci used to amplify *Mus spicilegus* DNA.

Locus	Annealing temp. (ºC)	Forward primer (5' end tagged with CAGTCGGGCGTCATCA)	Reverse primer
D15Mit16	52	AGACTCAGAGGGCAAAATAAAGC	TCGGCTTTTGTCTGTCTGTC
D4Mit166	52	AGTTTCCTTTCTCTTCTACTTG	AGGGCATAGGAAACTTTCAGG
D5Mit25	52	AACACACCTCCATACTGGTCG	GGCTAACTGAAATTGTTTTGTGC
D1Mit28	58	CACCCACTAATGCTTGGCTT	TTGAGACTAGAGCAACATGAAAGC
D2Mit372	58	GAAGACTGAGTCACAACTTC	CGGAAGTGGAGAAAGTTACC
D11Mit150	60	GGTCAGACACTGAGTGAAGATATAGC	TCCTCTGACACCCATAAGTTCA
D17Mit20	60	AGAACAGGACACCGGACATC	TCATAAGTAGGCACACCAATGC
D1Mit211	61	GTTATTCATCAAAATACAGATGGCC	TCTGCTGCTAAGTAGAATGAATGC
D10Mit86	61	TTTGCCTGTAACAAGCCAGA	TTGAGGCTATCAGTTTAAAATCC
D18Mit55	61	ACAGATGTTCCCCAGCATTC	TGAGTGTGAGATCAGCCTG
DXMit3	58	AAAAGGTCATGGCAAAAGGA	AGGAGAAAGTGCAGGGAGGT
DXMit5	58	CAACCTCTGAGCTCTCCCAC	TGTTGTCTAATTCCTTCAGGCA
DXMit22	60	CCATGCTCACAGGCACAC	CAGGCTGGGCTACAGAAGAC
DXMit23	60	GAGGATCATCAGCAAGCTCC	GCACTTCCTTTCCTAACACCC

mtDNA sequence analysis

DnaSP v. 5 (Librado & Rozas 2009) was used to calculate genetic diversity indices, and to collapse 238 sequences of 877bp from the mitochondrial control region into 36 unique haplotypes. We determined the most appropriate model for phylogenetic analyses using the

Akaike nformation Criterion in jModelTest 2.1 (Posada 2008). We then used the HKY++G model and default priors to assess the phylogeographic structure of M. spicilegus in MrBayes 3.2 (Ronquist & Huelsenbeck 2003), using the *M. macedonicus*, *M. cypriacus*, *M. spretus* and *M. musculus* as outgroups. MrBayes uses a Metropolis-coupled, Markov Chain Monte Carlo sampling approach to calculate Bayesian posterior probabilities. We ran 4 chains simultaneously for 10,000,000 generations in two independent runs, sampling trees every 500 generations. A burnin of 1,000,000 trees was carried out for each independent run, and a consensus tree with nodal posterior probabilities generated and displayed with FigTree 2.3.1 (Rambaut 2009). Using only the 36 unique *M. spicilegus* haplotypes (Table 2.1), several unrooted haplotype networks were constructed, including a 95% statistical parsimony network in TCS 2.21 (Clement et al. 2000), and a neighbor net and median-joining networks in SplitsTree 4 (Huson & Bryant 2006). We assessed genetic structure among geographic regions using the Analysis of Molecular Variance (AMOVA) in Arlequin 3.5 (Excoffier & Lischer 2010), with 10100 permutations to test for significance.

Microsatellite analysis

Using GenAlEx6 (Peakall & Smouse 2006), we checked that all 14 loci were not in linkage disequilibrium, and that allele frequencies did not depart significantly from Hardy-Weinberg Equilibrium. n addition, we tested for a pattern of isolation by distance using a Mantel test, testing for significance with 9,999 permutations. Arlequin 3.5 (Excoffier & Lischer 2010) was used to calculate pairwise Fst between populations, and assess the partitioning of genetic variation within and between populations using an AMOVA, with the sixteen populations grouped into three larger demes, color coded in Fig. 2.2. Population structure was also assessed in Structure 2.3 (Pritchard et al. 2000), assuming independent allele frequencies.

Structure infers the presence of distinct populations using a model-based Bayesian clustering method in which the user defines a number of populations (K), each characterized by a set of allele frequencies at each locus, and individuals are assigned probabilistically to populations based on their genotypes. Ten independent runs for each value of K were performed for K=1 to 16, with a burnin of 10,000, followed by 100, 000 MCMC iterations. We compared the consistency of results across the ten replicate runs at each value of K to check for MCMC convergence. We then used Structure Harvester (Earl & vonHoldt 2012) to determine the K that best fit the data, by calculating an *ad hoc* quantity, ΔK, from the second order rate of change of the likelihood function with respect to K (Evanno et al. 2005). A vast majority of samples (382 mice) were trapped in Bulgaria (Table 2.1), we repeated the Structure analysis on the Bulgarian populations only to see if that would reveal structure at a finer geographic scale. n addition, the GeneticStudio package (Dyer 2009) in R 2.15.1 (R Development Core Team 2012) was used to generate a population graph representing both within and among population genetic variance (Dyer & Nason 2004). Unlike F-statistics or AMOVAs that rely on summary statistics and pairwise population comparisons, the "Population Graphs" method uses a multivariate graph-theoretic approach that is free of *a priori* population arrangements, and simultaneously captures high-dimensional genetic covariance relationships among all populations.

Results

mtDNA sequences, phylogeography and geographic structure

Of the 238 *M. spicilegus* mtDNA control region sequences, we identified 36 unique haplotypes with 44 polymorphic sites, a mean nucleotide diversity of 0.01053 $\pm$ 0.00079, and a mean haplotype diversity of 0.937 $\pm$ 0.024.

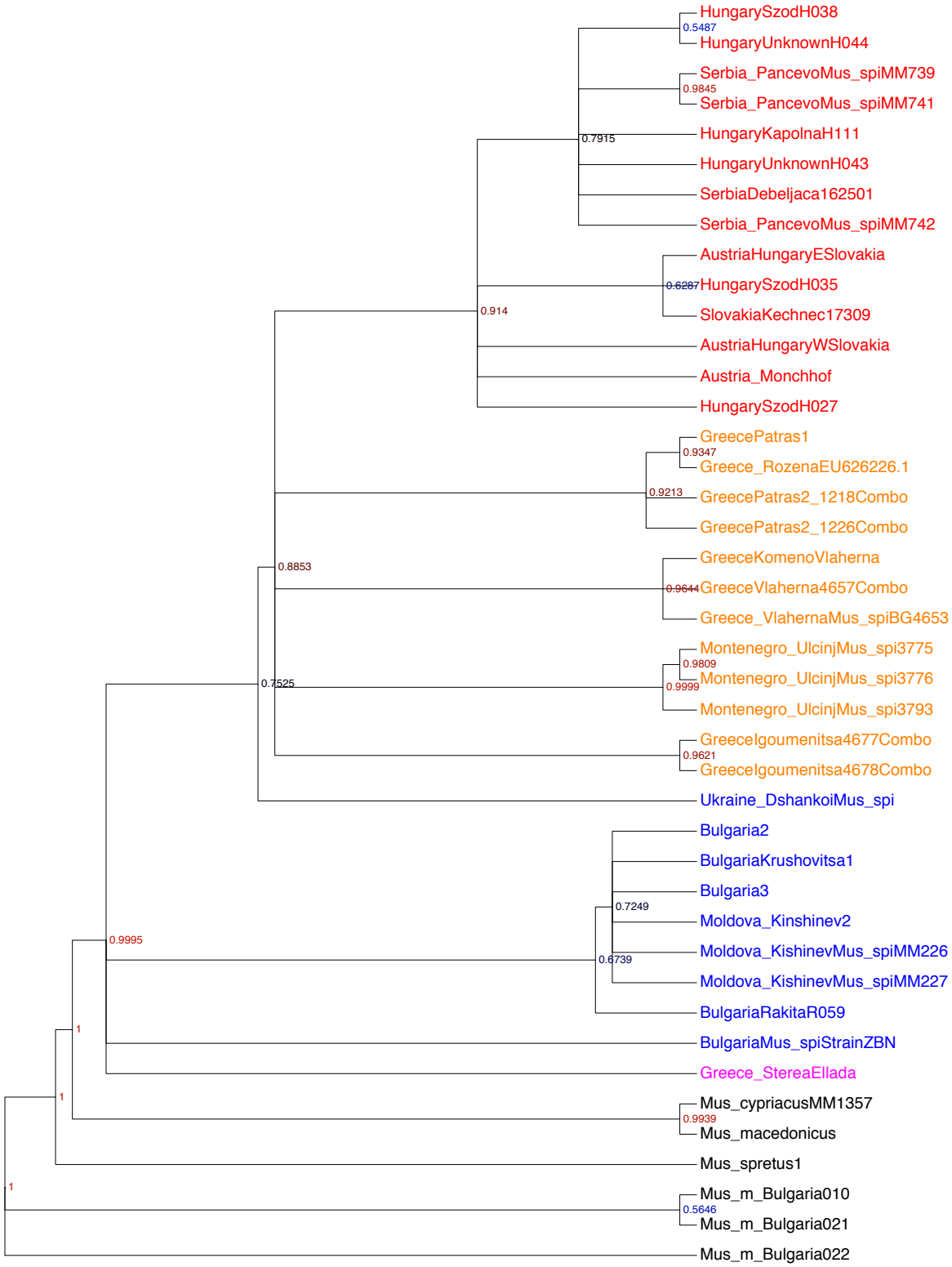


Figure 2.2 Bayesian tree of *Mus spicilegus*. Numbers on branches are posterior probabilities. Geographic populations are color coded corresponding to the map in Fig. 2.2. In red is the north-western clade, in blue the eastern clade, in yellow, *M. s. adriaticus* in the south, and in pink the recently discovered eastern Greek population from Sterea Ellada.

The Bayesian tree is largely unresolved, but the northern populations in Austria, Hungary, Slovakia and Serbia form a well-supported clade (posterior probability = 0.914). In the networks, all haplotypes tend to cluster by geographic location (Fig.s 2.3, 2.4, 2.5), with the exception of the Sterea Ellada population from Greece, which appears to cluster with the eastern *M. spicilegus* clade in both the neighbor net (Fig. 2.4) and median-joining (Fig. 2.5) networks.

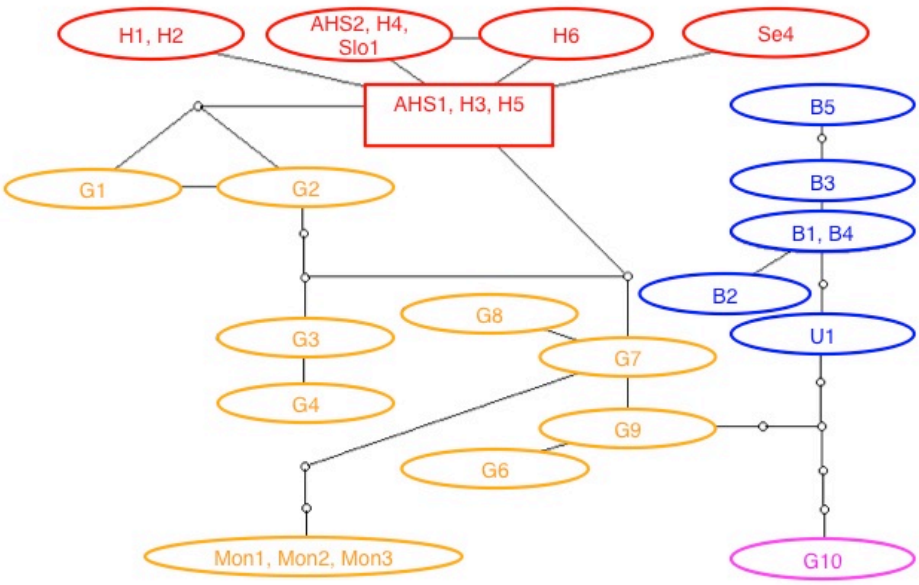


Figure 2.3 TCS statistical parsimony network. Haplotypes are shown in the ovals, and labeled by color according to sampling location. In red is the northwestern clade, in blue the eastern clade, in yellow, *M. s. adriaticus* in the south, and in pink the recently discovered eastern Greek population from Sterea Ellada. Mutational steps are represented by small circles between haplotypes.

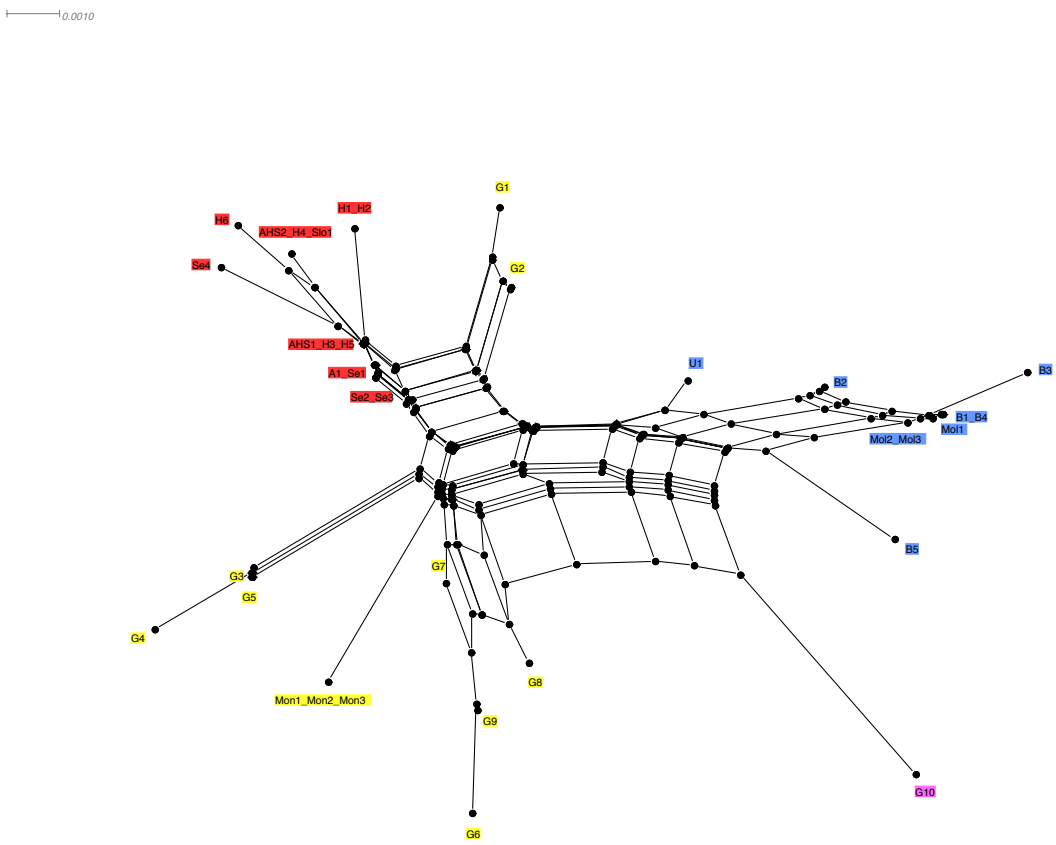


Figure 2.4. Neighbor Net tree from SplitsTree4. In red is the north-western clade, in blue the eastern clade, in yellow, *M. s. adriaticus* in the south, and in pink the recently discovered eastern Greek population from Sterea Ellada.

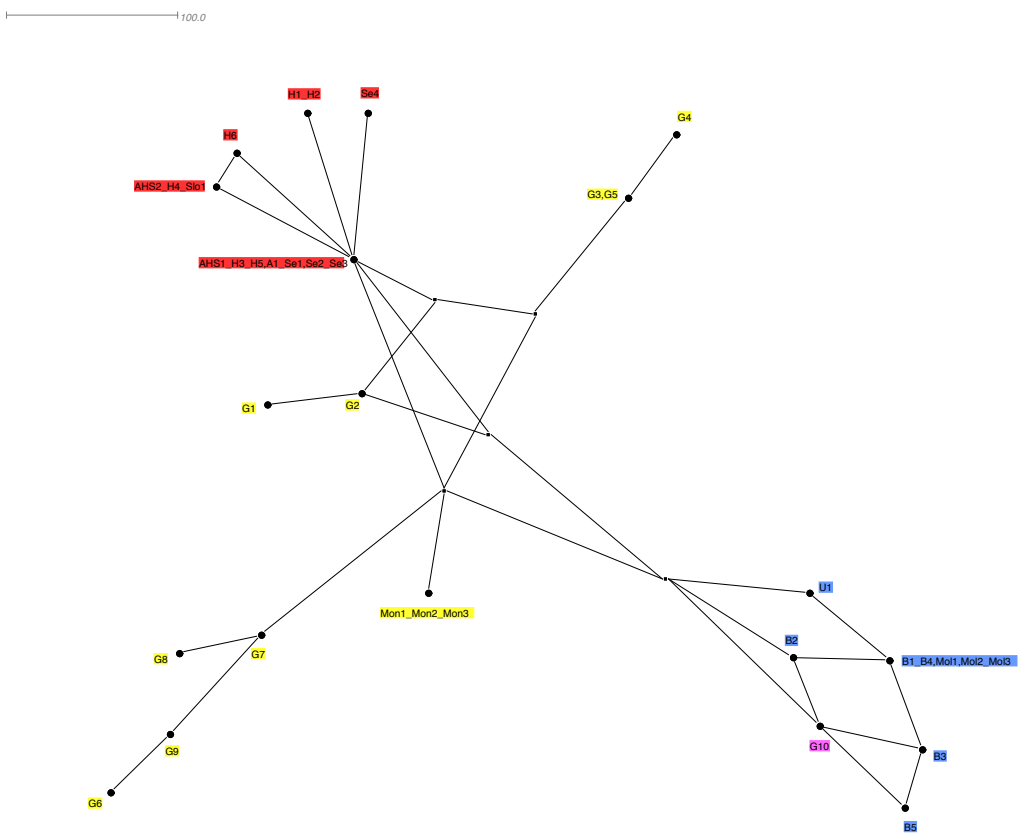


Figure 2.5. Median joining tree from SplitsTree4. In red is the north-western clade, in blue the eastern clade, in yellow, *M. s. adriaticus* in the south, and in pink the recently discovered eastern Greek population from Sterea Ellada.

The AMOVA analysis grouping Sterea Ellada haplotypes with the eastern European *M. spicilegus* showed a slightly higher percentage of variation among groups (42.91%) compared to 42.78% when Sterea Ellada was grouped with the *M. s. adriaticus* haplotypes (Table 2.3). In both population groupings, there was much higher variation among populations (~42%) than within populations (~15%).

Table 2.3. AMOVA results showing haplotype variation across groups, populations and within populations, depending on whether the eastern Greek Sterea Ellada haplotypes are grouped with the eastern *M. spicilegus* clade from Bulgaria, Moldova and Ukraine, or with the *M. s. adriaticus* subspecies in the rest of Greece. p <0.0001 for all values.

Source of variation	d.f	% total variation (Sterea Ellada + Eastern M. spicilegus)	% total variation (Sterea Ellada + *M.s. adriaticus*)
Among groups	2	42.91	42.78
Among populations within groups	5	42.14	42.24
Within populations	173	14.95	14.98

Microsatellites and population structure

Allelic variation ranged from 10-43 alleles per locus, and the average heterozygosity ranged from 0.37-0.865 (Table 2.4). The Mantel test did not yield a significant pattern of isolation by distance (Rxy = 0.042, p = 0.054).

Table 2.4 Summary statistics of 14 microsatellite loci in *Mus spicilegus*. Heterozygosity for the X-linked markers was calculated using only females.

Locus	Chromosome	n	Number of alleles	Range	H_E
D15Mit16	15	565	25	124-170	0.712
D4Mit166	4	556	29	178-294	0.744
D5Mit25	5	542	43	237-387	0.865
D1Mit28	1	574	16	108-158	0.640
D2Mit372	2	549	29	104-160	0.837
D11Mit150	11	569	17	130-233	0.877
D17Mit20	17	570	25	120-246	0.731
D1Mit211	1	575	10	118-168	0.372
D10Mit86	10	570	15	154-214	0.636
D18Mit55	18	570	32	157-213	0.805
DXMit3	X	254	18	158-216	0.633
DXMit5	X	252	32	142-224	0.845
DXMit22	X	240	19	240-280	0.710
DXMit23	X	240	19	247-281	0.647

Nevertheless, populations are well-differentiated, as all the pairwise F_{ST} values are significant (Table 2.5), and an AMOVA grouping the populations in the same geographic groupings as displayed in Fig. 2.1 shows a small but significant percentage of total genetic variance segregating among groups and populations (Table 2.6). The mean pairwise F_{ST} (Table 2.5) across all Greek *M. s. adriaticus* and Bulgarian populations is 0.149, slightly lower than the

mean pairwise F_{ST} across all northern and Bulgarian populations of *M. spicilegus* (F_{ST} =0.184), while the largest difference is between the geographically most distant populations in the north and *M. s. adriaticus* in Greece (F_{ST} = 0.323).

Table 2.5 Pairwise population F_{ST} values. All values are significant ($p < 0.05$), based on 99 permutations. Geographic populations are color coded in the same scheme as shown in the map in Fig. 2.2.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0.000															
2	0.072	0.000														
3	0.149	0.135	0.000													
4	0.136	0.130	0.035	0.000												
5	0.159	0.134	0.061	0.084	0.000											
6	0.252	0.238	0.167	0.129	0.161	0.000										
7	0.404	0.363	0.261	0.224	0.262	0.129	0.000									
8	0.179	0.193	0.136	0.159	0.121	0.235	0.269	0.000								
9	0.183	0.211	0.137	0.156	0.143	0.277	0.326	0.110	0.000							
10	0.163	0.192	0.139	0.167	0.140	0.247	0.254	0.055	0.065	0.000						
11	0.147	0.168	0.123	0.134	0.112	0.197	0.223	0.043	0.052	0.012	0.000					
12	0.172	0.190	0.151	0.170	0.153	0.224	0.257	0.065	0.083	0.035	0.027	0.000				
13	0.344	0.362	0.266	0.293	0.252	0.385	0.510	0.128	0.179	0.134	0.099	0.129	0.000			
14	0.258	0.300	0.200	0.227	0.186	0.335	0.423	0.119	0.137	0.111	0.071	0.113	0.164	0.000		
15	0.445	0.409	0.330	0.338	0.304	0.445	0.602	0.213	0.290	0.209	0.174	0.201	0.354	0.230	0.000	
16	0.258	0.273	0.198	0.222	0.179	0.314	0.376	0.152	0.172	0.132	0.102	0.114	0.201	0.159	0.263	0.000

Table 2.6 AMOVA and F-statistics results showing variance in microsatellite allele frequencies across groups, populations, within populations and within individuals. $p < 0.0001$ for all values.

Source of variation	d.f	% total variation	Fixation indices
Among groups	2	9.56	F_{CT} = 0.240
Among populations within groups	13	5.59	F_{SC} = 0.062
Within populations	563	20.39	F_{IS} = 0.240
Within individuals	579	64.47	F_{IT} = 0.355

Consistent with the mean pairwise F_{ST} values across groups of populations, Structure groups the populations into two main groups. However these groups do not correspond to the current taxonomic classifications of *M. spicilegus* and *M. s. adriaticus*. Rather, the Greek *M. s.*

adriaticus cluster with Bulgarian *M. spicilegus*, in a separate "group" from the northern *M. spicilegus* in Hungary, Serbia and Slovakia (Fig 2.6a). Within Bulgaria, from which most of the samples were collected, the number of clusters that best fit the data was six, suggesting that there is additional structure within this smaller geographic range. The most geographically distant population, Srebarna (population 8 in Fig 2.6b), in northeastern Bulgaria, is 230km from the other populations, and shows the clearest evidence of being a genetically distinct cluster. The next most distant population, Knezha, in northwestern Bulgaria (population 12 in Fig. 2.6b), is 30km from the other populations, and shows slight evidence of forming a different genetic cluster. The remaining populations (9-11 in Fig. 2.6b) were collected within 5-15km from each other, so a lack of obvious genetic structure corresponding to geographic structure is unsurprising.

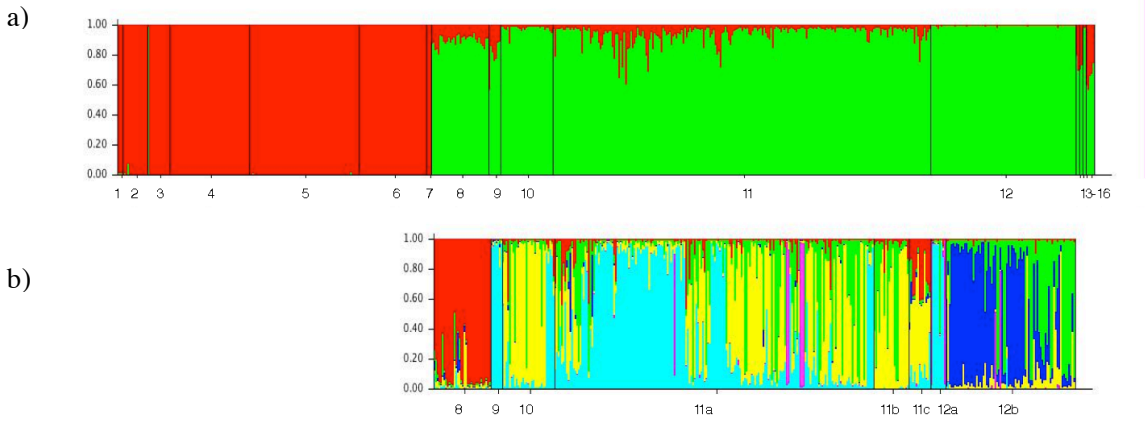


Figure 2.6 Structure results for microsatellites, where each vertical bar represents an individual and the proportion of each genotype that is assigned to a particular population cluster is shown in a different color. The numbers below the represent the geographic location individuals were sampled from. a) From 16 populations (defined in Table 2.1). Populations are arranged from north to south, with the red showing populations corresponding to the northern locations in the other figures (Fig. 2.1), and green, the eastern (Bulgarian) and southern (Greek) populations. b) From Bulgarian populations only, with individuals arranged in the same order as in 2.6a.

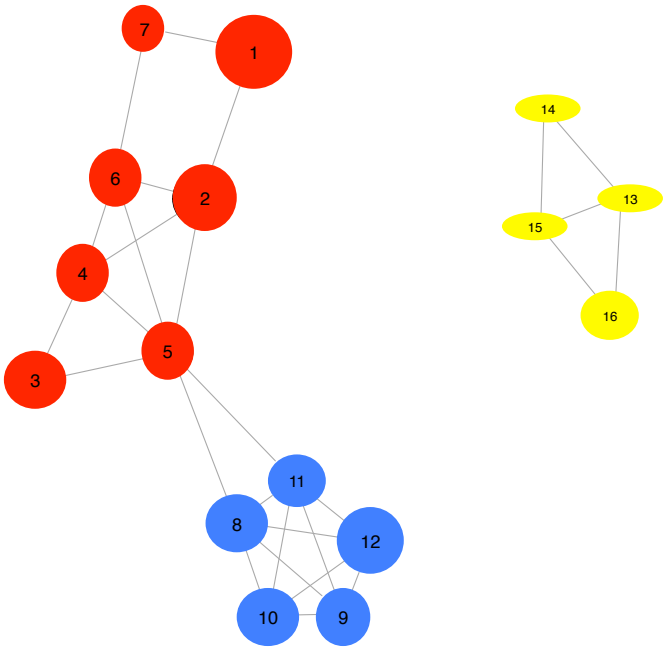


Figure.2.7 Population Graph representing genetic relationships among the three main geographic clades of *M. spicilegus*. In red is the northwestern clade, in blue the eastern clade, in yellow, *M. m. adriaticus* in the south. The differences in node size reflect differences in within population genetic variability, and the edge lengths represent genetic variation among populations.

Contradicting the F_{ST} and Structure results, the "Population Graphs" analysis on microsatellite data agrees with current taxonomy, and shows that the *M. spicilegus* populations in the north and east are disconnected from the *M. s. adriaticus* populations in the south (Fig. 2.7).

Discussion

The Bayesian tree using the mitochondrial control region shows that while *M. spicilegus* forms a clade (posterior probability = 0.999), the relationships of the geographically and morphologically disjoint *M. s. adriaticus* and the recently discovered Sterea Ellada populations

remain unresolved (Fig. 2.2). Interestingly, the northwestern populations, from Austria, Hungary, Serbia, Slovakia form a clade (posterior probability = 0.914), whereas the *M. s. adriaticus* sequences are possibly nested within the *M. spicilegus* tree, next to the northwestern populations (posterior probability = 0.885), with samples from Sterea Ellada and the northeastern populations as outgroups. Based on this poorly resolved tree, the question of which route *M. spicilegus* took colonizing its current range remains unresolved. Sequencing other populations and/or loci should help to determine if the *M. spicilegus* travelled over the north of the Black Sea, or took a more southerly route, and was subsequently pushed northwards by its sister species, *M. macedonicus*.

All three haplotype networks (Fig.s 2.3-2.5) suggest that the recently discovered Sterea Ellada haplotype from eastern Greece is quite distinct from other *M. spicilegus* haplotypes. The neighbor net (Fig. 2.4) and median joining tree (Fig. 2.5) in particular, also suggest that the Sterea Ellada population is more similar to the northeastern *M. spicilegus*, than to the geographically closer *M. s. adriaticus* populations. This result is consistent with the findings of Mitsainas and colleagues (2009), who place this haplotype at the base of a *M. spicilegus* tree.

Consistent with this pattern from the haplotype networks, the AMOVA results also suggest that the Sterea Ellada haplotype is slightly more similar to those of northeastern *M. spicilegus* than to the Greek *M. s. adriaticus* haplotypes, as there is a slightly higher percentage of variation among groups (42.91%) when Sterea Ellada is grouped with the northeastern *M. spicilegus*, compared to 42.78% when Sterea Ellada is grouped with *M. s. adriaticus* (Table 2.3). As Sterea Ellada is represented by a single haplotype from GenBank, additional sampling

from this area, and sequences from other loci would greatly help to determine how this recently discovered population of *M. spicilegus* is related to the rest of the species.

With the addition of new samples from the eastern range of the northern *M. spicilegus* distribution, neither the mitochondrial sequence nor the microsatellite data strongly support a taxonomic split between *M. spicilegus* and *M. s. adriaticus*. In the phylogeny (Fig. 2.2), and all three haplotype networks (Fig.s 2.3-2.5), *M. s. adriaticus* haplotypes (in yellow) appear to be intermediate between the two northern *M. spicilegus* clades (in red and blue). Similarly, mean pairwise F_{ST} (Table 2.5) comparing all Greek *M. s. adriaticus* to northeastern populations is 0.149, slightly lower than the mean pairwise F_{ST} comparing northwestern and northeastern populations of *M. spicilegus* (F_{ST} =0.184), suggesting that *M. s. adriaticus* populations are more similar to northeastern *M. spicilegus* than the two *M. spicilegus* populations are to each other. Finally, the same pattern is suggested by a Bayesian clustering of microsatellite genotypes in Structure, which assigns northeastern *M. spicilegus* and *M. s. adriaticus* to a single cluster distinct from the northwestern *M. spicilegus* populations (in red in Fig. 2.6).

By contrast, the graph theoretic analysis using microsatellites does indeed group the northwestern and northeastern *M. spicilegus* populations, with the *M. s. adriaticus* populations forming a separate network, which does support the current taxonomic division, and is consistent with the distinct geographic ranges of these two subspecies (Fig. 2.1). A possible explanation for this ambiguity is that the northeastern *M. spicilegus* populations diverged relatively recently from *M. s. adriaticus*, from which they currently appear to be geographically isolated. Consequently, the microsatellite analyses shows a clear difference between the most

geographically distant northwestern *M. spicilegus* populations and *M. s. adriaticus* in the south, with the geographically intermediate northeastern *M. spicilegus* also genetically intermediate. This interpretation is also consistent with an insignificant pattern of isolation by distance (Rxy = 0.042, p = 0.054) (Wright 1943), suggesting that there has been substantial gene flow across populations in this species, or a very recent population expansion, probably out of glacial refugia (Auffray & Britton-Davidian 2012) .

Also consistent with a lack of isolation by distance is the general lack of structure both across the entire species range (Fig. 2.6a), and within the more densely sampled Bulgarian populations (Fig. 2.6b). The general lack of significant population structure in microsatellite variation is further supported by the low levels of variation segregating across populations (5.59%) and groups (9.56%) in an AMOVA (Table 2.6), and by the poorly resolved phylogeny (Fig. 2.2). A general lack of structure could be the result of a recent expansion into a relatively small species range, compared to its free-living sister species, *M. macedonicus*, which forms two deeply-diverged subspecies between populations in Israel and those from the rest of the species range (Orth et al. 2002). These results could also reflect the ecology of mound-building mice, suggesting that as non-commensals, they tend to disperse further than house mice, which do show strong within-species differentiation (Suzuki & Aplin 2012). The next chapter will investigate dispersal in this species at a finer geographic scale.

CHAPTER 3

Kinship and dispersal within populations of mound-building mice (*Mus spicilegus*)

Summary

Several aspects of *Mus spicilegus* biology including social monogamy, large litters and the shared construction of mounds make the species appear pre-adapted for cooperative breeding. However, the genetic mating system, dispersal patterns and kinship during the breeding season remain elusive. Using a combination of trapping methods and microsatellite genotyping, we provide the first evidence that *M. spicilegus* is not genetically monogamous, and that litters from populations over 200km apart have 2-3 fathers. Genetic relatedness between mice associated with the same mound is higher in fall, the mound-building season, than in spring, when breeding begins. There is no sex difference in relatedness within mounds, and we find no other evidence of a strong sex bias in dispersal. However at very small spatial scales within a hectare, we find that adult males are more likely to associate with kin of either sex in spring, but particularly male relatives. I also found that the sex ratio was male-biased in spring. In contrast, relatedness between female adults caught at the same trap in spring is no greater than the average pairwise relatedness between females caught anywhere within the hectare. I suggest that clustering spatially with male kin is a strategy that socially monogamous males adopt to offset the costs of extra-pair paternity when the population density of males is unusually high.

Introduction

Limiting natal dispersal by at least one sex is often a necessary precursor to the evolution of cooperative breeding, which is defined by the presence of non-reproductive caretakers in a group (Emlen 1978). In most birds, dispersal is female-biased, while the opposite is true for most mammals (Greenwood 1980). Mating systems and competition for resources can be used to explain these broad differences in which sex disperses further. The argument is that the mating system in most mammals involves female-defense polygyny, and females experience more competition for resources than do males, thereby selecting for female-biased philopatry among females that benefit from familiarity with their territories. In contrast, most birds are socially monogamous, and males are under selection to defend the best territories, rather than females, so in birds, it is sons that disperse less because of the selective advantages of territory defense (Greenwood 1980). More recently, comparative analyses have also identified monogamy (Lukas & Clutton-Brock 2012) and multiple-offspring litters (Lukas & Clutton-Brock 2012) as prerequisites of cooperative breeding in mammals.

Another common precursor of cooperative breeding is the communal construction of a shelter (Costa 2006). This is because once built, the shelter provides a common good, and a defensible resource. Naked mole rats are the ultimate example of cooperative breeding in mammals (Jarvis 1981); however, a range of other rodents also construct shared shelters with kin (King 1955; Koprowski 1996; Blumstein & Armitage 1999; Ebensperger & Blumstein 2006). One of the shared benefits of a group shelter is warmth, and given their small body size, huddling is an excellent way for rodents to minimize heat loss in cold weather. Many examples of huddling for warmth tend to involve kin, although not all individuals in a

huddle are related (Schradin et al. 2006; Thorington & Weigl 2011). Shared nests are also commonly used for breeding, often with female kin (Manning et al. 1995; Blumstein & Armitage 1999; Ebensperger 2001).

To investigate the role of cooperative construction, monogamy and dispersal in the evolution of cooperative breeding, this study focuses on what Darwin (1859) would have called an incipient form. The mound-building mouse, *Mus spicilegus*, is named for its ability to construct large overwintering mounds (Muntyanu 1990). In contrast to its close relative the house mouse, which tends to form communally nesting female groups (Manning et al. 1995), *M. spicilegus* only lives in groups during the winter, and subsequently disperses to breed. Both field and laboratory evidence strongly suggest that this species is socially monogamous (Patris & Baudoin 1998, 2000; Dobson & Baudoin 2002; Simeonovska-Nikolova 2007). In addition, females living together appear to suppress reproduction in each other (Féron & Gheusi 2003; Gouat & Féron 2005). In short, *M. spicilegus* fulfills several known criteria required for cooperative breeding to evolve, including communal construction, a large litter size (like most mice), and social monogamy with evidence for a social suppression of reproduction between females.

Existing evidence on kinship, dispersal and the genetic mating system of *M. spicilegus* is tantalizingly incomplete. We know that mounds are inhabited by the litters of at least two pairs of genetically related parents (Garza et al. 1997), but do not know when these groups form, or how they disperse in the spring. Indeed, the dispersal patterns of *M. spicilegus* are unknown, and sex ratios from trapping studies at different times of year, using different methods, have yielded highly varied results (Table 1.2), making it difficult to infer either

mating system or dispersal patterns from sex ratios in this species. Finally, evidence from one mark-recapture study suggests that *M. spicilegus* is capable of breeding polygynously in the wild (Gouat et al. 2003), while evidence of high levels of sperm competition (Gomendio et al. 2006; Gómez Montoto et al. 2011b) suggest that polyandry has been common in the recent evolutionary past of this socially monogamous species.

This study investigates aspects of *M. spicilegus* life history that have yet to be determined, but are necessary to understand why this species has not evolved into a cooperative breeder.

1. Are *M. spicilegus* genetically as well as socially monogamous? I predict that females actually mate with more than one male, because of the strikingly large relative testis size in this species, suggesting high levels of sperm competition (Gomendio et al. 2006; Gómez Montoto et al. 2011a).
2. Sex ratios can influence competition for mates and other resources. I expect that the sex ratios of *M. spicilegus* at different seasons could predict the mating system or a sex-bias in dispersal.
3. Most mammals have male-biased dispersal; however, given that *M. spicilegus* is socially monogamous, I predict that in this species, dispersal could be female-biased.
4. How does kinship differ between the mound-building season in fall, and the breeding seasons in spring and summer? I predict that genetic relatives are in closer proximity in fall than in the other seasons.

Methods

Study area and DNA sampling

In the spring (April) and autumn (Sep-Oct) of 2007-09, I trapped 229 mice from 67 mounds from 8 populations in Hungary and Bulgaria (Table 3.1, Fig. 3.1). Collapsible Sherman traps (3 x 3 x 9 inches) were baited with rolled oats, and placed around mounds, near entrance holes to the mounds. Three pregnant females were trapped and sacrificed in April 2009, and their embryos (N= 8, 7, 5) dissected and stored individually in tubes of 99% ethanol to minimize cross-contamination before DNA was extracted from tissue from the center of each embryo.

Table 3.1 Locations and numbers of mice trapped from specific mounds in Hungary and Bulgaria. Populations with numbers in superscript are the ones where pregnant females were trapped, and the numbers indicate sample sizes.

Country	Population	Latitude ºN	Longitude ºE	Season	Number of mounds	Number of males	Number of females	Total number of mice
Bulgaria	Krushovitsa	43.348975	24.41527	Fall	4	5	2	7
	Knezha[2]	43.497984	24.08117	Spring	12	12	24	36
	Srebarna[1]	44.094442	27.06402	Spring	9	16	11	27
	Telish 2	43.290900	24.20605	Fall	7	16	5	21
	Telish 3	43.361317	24.39213	Spring	5	11	2	13
Hungary	Kapolna	47.759113	20.24708	Fall	12	31	35	66
	Szöd	47.724664	19.17139	Fall	12	25	22	47
	Unknown	NA	NA	Fall	6	5	7	12

Figure 3.1 Map showing sampling locations for mounds (black stars), and the location of the long-term field site and trapping grid (white star).

In addition, I established a 100m by 100m trapping grid near the village of Telish in Bulgaria (43.327 ºN, 24.261 ºE), with a trap every 10m (Fig.s 3.1, 3.2). Mice were also trapped in Sherman traps baited with rolled oats. A total of 7 trapping sessions, each 8-10 nights long took place in the spring (April), summer (July) and fall (late August-early September) of 2008 and 2009, and late fall (October) 2009. Mice were individually marked by toe clipping, and toe clips and tail tips preserved in 99% ethanol for DNA extraction. I also recorded the sex,

reproductive condition, weight, body length, tail length, hind-foot length, ear length and testis length (for mature males) for each capture. Finally, all the mounds in the trapping grid were mapped and measured for their general shape, maximum height above the ground, length and width. I also recorded if the mounds were being used, or had been abandoned, by using the presence of freshly turned earth and feces at the entrance holes to the mounds as indicators of an active mound. The Institutional Animal Care and Use Committee of the Faculty of Arts and Sciences, Harvard University, approved our field trapping methods (Protocol No. 27-17).

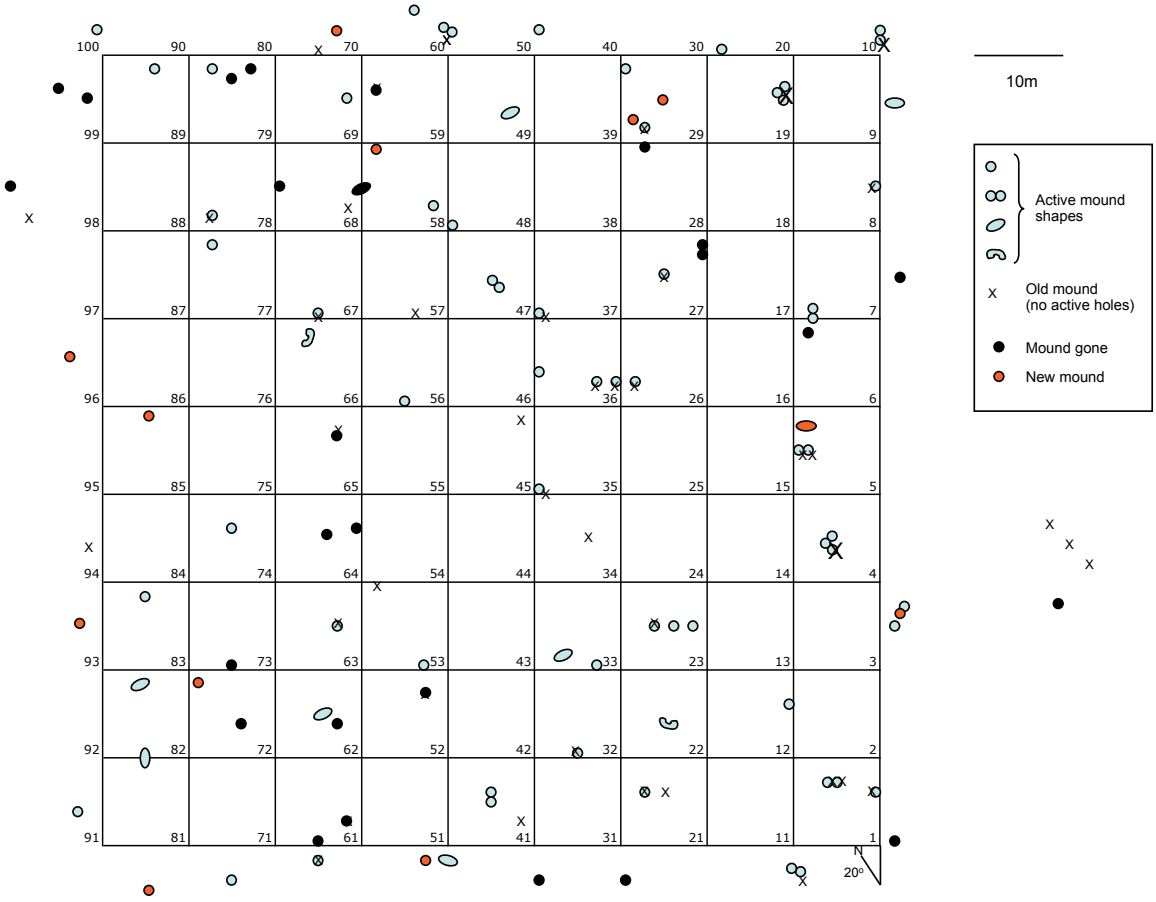


Figure 3.2 Trapping grid near Telish, Bulgaria in 2009. Each number represents a trap, set 10m apart. Mounds are represented by symbols representing the shape of the mound, and color coded to show mounds active in 2009 that were first recorded in 2008 (blue), inactive mounds (cross), mounds that were present in 2008, but absent in 2009 (black), and mounds newly recorded in 2009 (orange).

DNA extraction and microsatellite genotyping

I extracted genomic DNA from tail tips and toe clippings using a Qiagen DNeasy Blood and Tissue Kit (Qiagen). I then amplified ten autosomal and four X-linked microsatellites using published primers (Garza et al. 1997; Schalkwyk et al. 1999). PCR reactions were amplified in a 15µl reaction containing 2.5µl of 10x reaction buffer, 0.3µl of 10mM dNTPs, 0.3µl of 50mM $MgSO_4$, 0.15µl of Amplitaq DNA polymerase (Applied Biosystems) and 2.0µl of 30-40ng/µl genomic DNA. To each reaction, I also added 0.54µl of a primer tag labeled with the fluorescent dye 6-FAM (Applied Biosystems) that would bind to 0.06µl of the forward primer tagged on the 5' end with a complementary CAG sequence (CAGTCGGGCGTCATCA), and 0.6µl of the reverse primer. All three primers were at the same concentration of 10µM. I used an Eppendorf Mastercycler ep (Eppendorf) for the reactions with one cycle of 3min at 94ºC, followed by 40 cycles of 30s at 94ºC, 45s at an optimized annealing temperature (Table 2.2) and 60s at 72ºC, and a final cycle for 10min at 72ºC. To visualize the microsatellites, 3µl of each PCR product was then combined with 0.3µl of the internal size standard ROX 400HD (Applied Biosystems) and 20µl of formamide, and run in an ABI 3730xl Genetic Analyzer (Applied Biosystems). To minimize errors, samples that were ambiguous were re-scored from new PCR reactions. Individuals genotyped for fewer than 8 loci were excluded from the analyses. Genotypes were scored using Peak Scanner 2.0 (Applied Biosystems).

Statistical analyses

Apart from relatedness calculations mentioned below, I used R 2.15.1 (R Development Core Team 2012) for statistics. To see if littermates had more than one father, I used an "allele counting method" by comparing the known maternal genotype to offspring genotypes for

both X and autosomal loci. X-linked microsatellite loci were especially useful, as each father would only have one X chromosome to pass on to his daughters, so I could use the number of paternal X alleles in female littermates to determine a minimum number of fathers (Garza et al. 1997; Poteaux et al. 2008).

I used exact binomial tests to calculate a deviation from an expected sex ratio of 50:50 for every season. I also wanted to see if one sex is more likely to be re-trapped in the same season. To do this, I used Wilcoxon rank-sum tests to compare the number of recaptured males to the number of recaptured females for each season. To see if one sex is more likely to occupy a larger space than the other, I used trapping data to calculate two distance measures for all the mice caught in more than one trap in a season. First, I calculated the maximum distance spanned by the traps that an individual mouse was caught at in a single season, next, I calculated a distance based on the mean of all the nearest-neighbor distances between traps for a mouse in a given season. I used Wilcoxon rank-sum tests to see if either of these distance measures differed significantly between the sexes for any trapping season.

I used the Relatedness 5.0 (Queller & Goodnight 1989) to estimate average pairwise relatedness for two datasets collected by trapping mice around mounds in different populations, and in a trapping grid. Significance was calculated by jackknifing across loci. For both datasets, I compared average pairwise relatedness between members of the same sex within and across seasons. For mice trapped around mounds, I compared relatedness within and among mounds. For mice from the trapping grid, I also calculated average relatedness within and across sexes of immature and mature individuals caught in the same trap in the

same season. Finally, I calculated the average pairwise relatedness of the five males and five females that were re-trapped across years.

For the trapping grid that was monitored over two years, I tested for a correlation between pairwise relatedness and geographic distance between mice trapped in the same season. Two measures of relatedness were used: Queller-Goodnight pairwise relatedness (Queller & Goodnight 1989) was calculated in GenAlEx6 (Peakall & Smouse 2006), and a maximum likelihood estimate that is independent of background allele frequencies, was calculated in ML-Relate (Kalinowski et al. 2006). Geographic distance was calculated for each pair of mice trapped in the same season using R 2.15.1 (R Development Core Team 2012), which I also used to perform a Pearson's correlation.

Results

Multiple paternity in Mus spicilegus

By genotyping the unborn litters of three pregnant mice, I found evidence of multiple paternity in all three litters. There were 8, 7 and 5 embryos per litter respectively, and in each litter the female embryos had two to three unique alleles in addition to their mother's alleles at X-linked loci. An exclusion test of paternity, given both offspring and maternal genotypes supports the hypothesis that each litter had two-three fathers.

Trapping data across seasons within a single population

Over the course of the seven trapping seasons and two years, 183 mice (111 males and 72 females), with more mice caught in 2008 than in 2009 (Fig. 3.3). A possible reason for this, is that there were 85 active mounds in 2008, and only 51 active mounds in 2009. Significantly

more males were trapped in spring 2008 (54 males: 28 females; exact binomial test, p = 0.005), whereas there is no significant sex ratio bias in any of the subsequent trapping seasons.

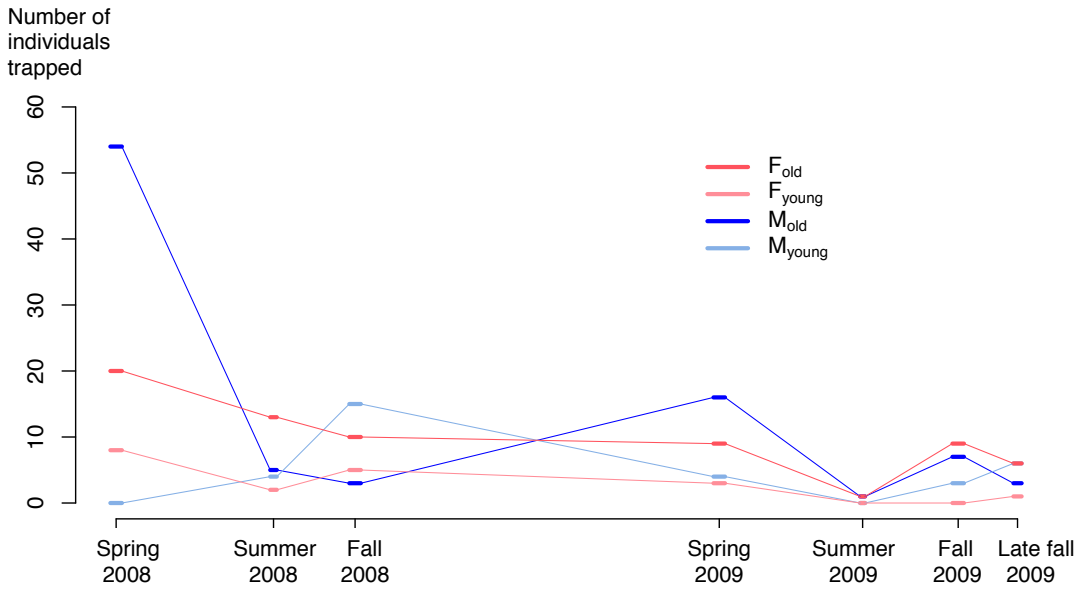


Figure 3.3 Number of individual mice trapped in all seven seasons. Trapping seasons are on the horizontal axis, and numbers of mice trapped on the vertical axis. Mice are color coded by sex and maturity. In general, more mice were trapped in 2008 than in 2009. More mature males are trapped in spring than any other season, while the most immature males are trapped in fall.

In total, I made 468 captures, and 111 of the mice were trapped more than once (Fig. 3.4). There is no significant difference in recaptures for males and females, except in fall 2009, when females were more likely to be re-trapped than were males (Wilcoxon rank-sum test; Z = 2.681, p = 0.007). There is also no significant sex difference in the distances travelled by mice within seasons or across all seasons for either distance measure.

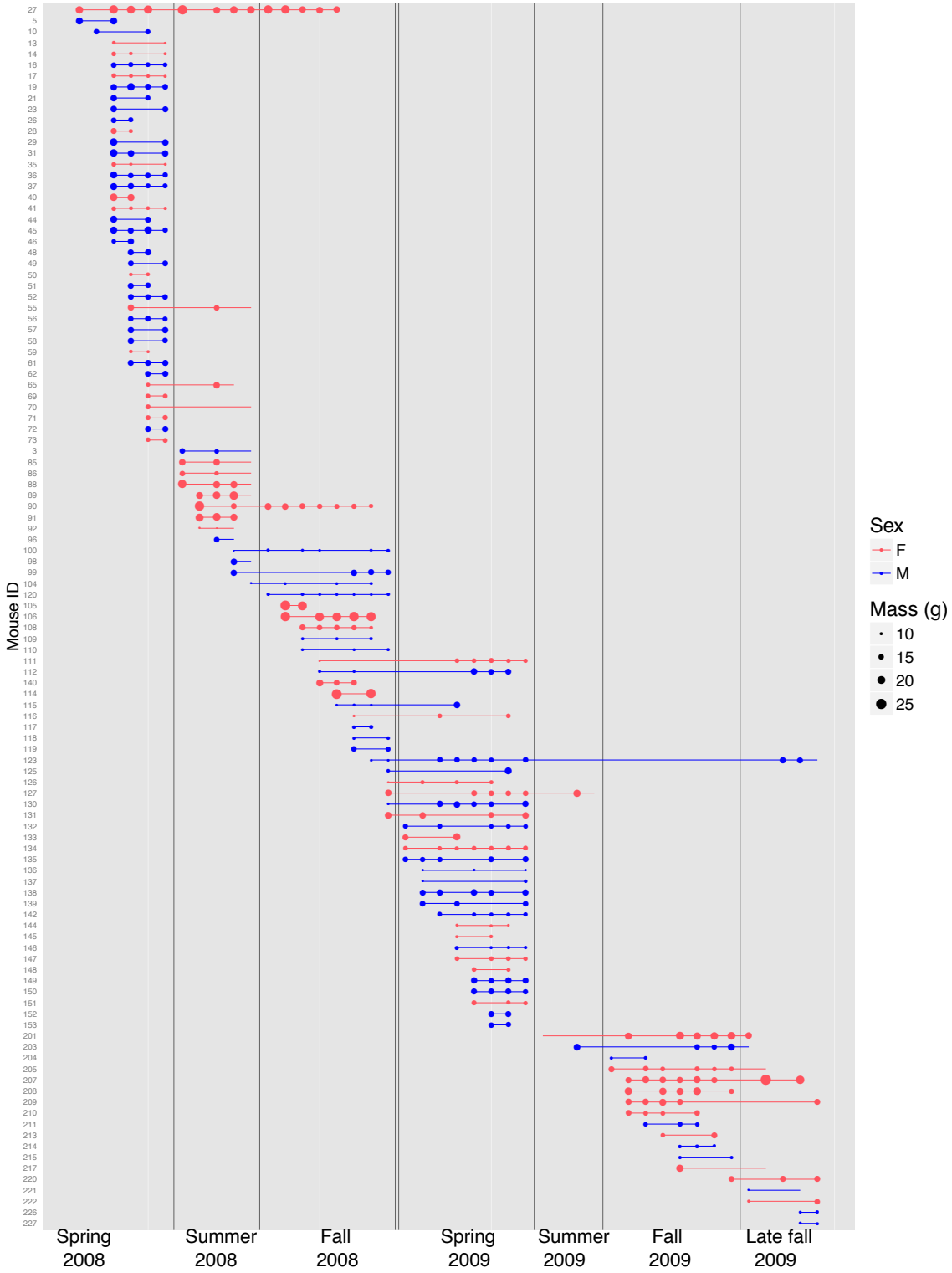


Figure 3.4 All mouse recaptures over time. Each row represents an individual that has been caught more than once. Vertical black lines represent boundaries between seasons, the double line, a between-year boundary. Circles represent each capture, and the size of the circle, the weight of the mouse. Red circles are females, blue, males.

Relatedness within and across mounds

I found that relatedness within populations (0.136 ± 0.014) is significantly higher than across populations (R-difference = 0.0993 ± 0.014, p = 0.0001). In turn, relatedness within mounds within populations (0.285 ± 0.015) is significantly higher than among mounds in the same population (R-difference = 0.1909 ±0.019, p = 0.0001). When broken down by season, average relatedness within mounds is significantly higher in fall (0.3228 ± 0.018) than in spring (0.208 ±0.022). None of these comparisons revealed a significant sex difference in relatedness (Fig. 3.5).

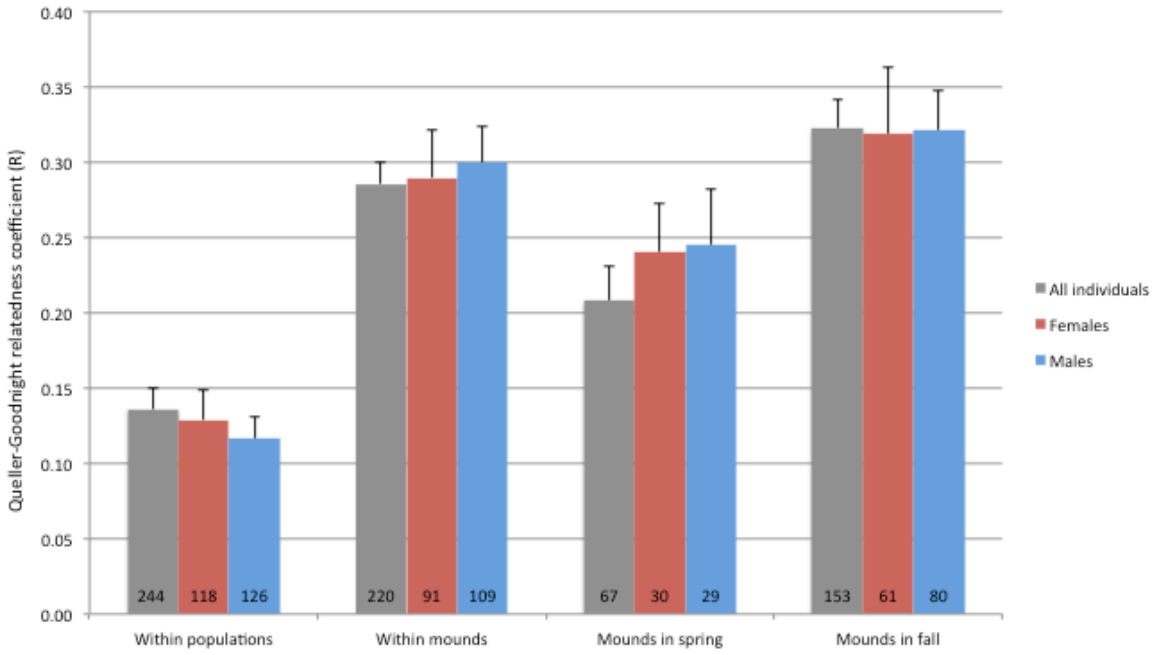


Figure 3.5 Average coefficients of Queller-Goodnight relatedness (R) within populations and within mounds in spring and fall. Standard errors were calculated by jackknifing across all 14 loci. Sample sizes are at the bottom of each bar. R is significantly greater within mounds than within populations, and significantly greater within mounds in fall than in spring for all individuals, all males and all females (P> 0.0001).

Relatedness within a single population across space and time

In contrast to relatedness calculations from trapping around mounds, mice trapped in the same location during the same trapping season showed a sex-bias in relatedness (Fig. 3.6). While there was no significant difference in average pairwise relatedness for all individuals, within males, within females, or between males and females trapped in the same season, female-female relatedness was significantly lower than other pairwise comparisons for individuals caught in the same trap. Females caught in the same trap in spring had an average pairwise relatedness of 0.0538 ± 0.05, whereas male-male pairs caught in the same trap had a relatedness of 0.1947 ± 0.06, which is significantly greater than the pairwise relatedness between males and females caught in the same trap (0.1462 ± 0.03, p = 0.01). In fall, females caught in the same trap had a pairwise relatedness of 0.0206 ± 0.06, again significantly less than the average relatedness between males (R = 0.1665 ± 0.06), or between males and females caught in the same trap (R = 0.1787 ± 0.03, p 0.0001). There is no significant difference between male-male and male-female relatedness for the same trap in fall.

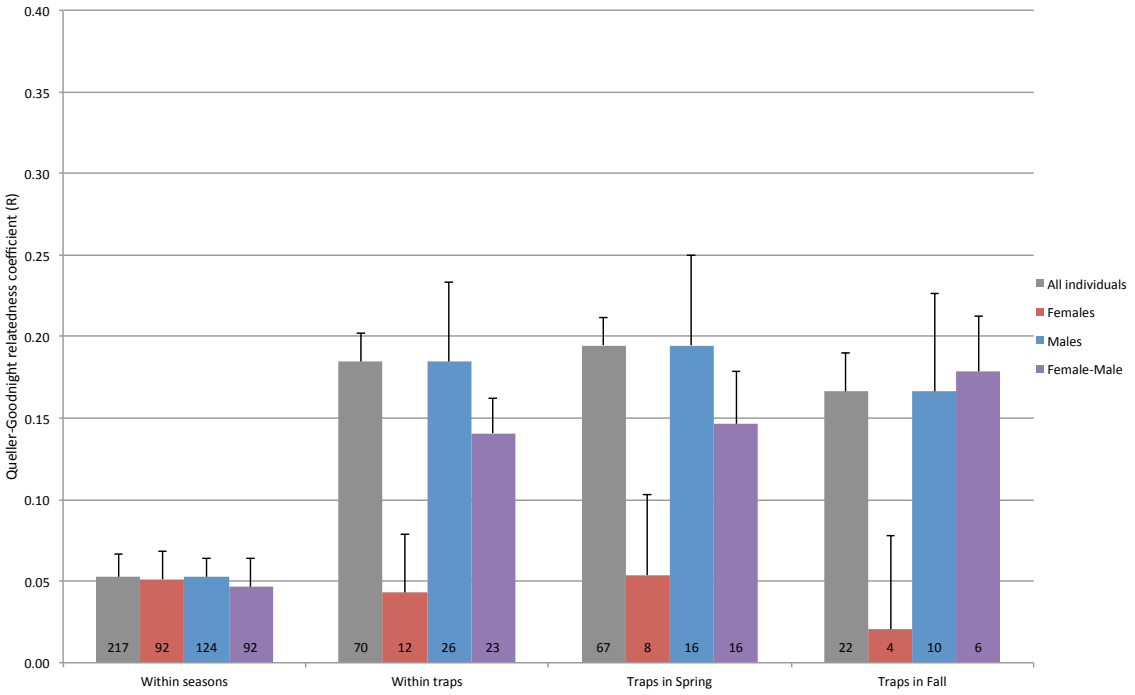


Figure 3.6 Average coefficients of Queller-Goodnight relatedness (R) within a single population within seasons and within traps in spring and fall. Standard errors were calculated by jackknifing across all 14 loci. Sample sizes are at the bottom of each bar. R is significantly greater within traps than among traps, with the exception of pairwise female relatedness (P> 0.0001).

To determine if mature or immature individuals were driving these sex-biases in relatedness, I repeated the calculations for mice weighing less than 12g and weighing 12g or more, as none of the mice under 12g were reproductively mature (Table 3.2). Due to a limited sample size, I could not rigorously perform all the comparisons for all the age and sex categories. However, I did find that the sex-differences in relatedness within traps, within seasons, are driven by a low relatedness between older females within traps in spring ($R = 0.062 \pm 0.05$), and a low relatedness between younger females within traps in the fall ($R = 0.0206 \pm 0.06$). In contrast, relatedness between older males within traps in spring was 0.1654 ± 0.05, while the average relatedness between younger males in fall was 0.3538 ± 0.09 (Table 3.2).

Table 3.2 Average pairwise relatedness for younger and older mice within the trapping grid. Standard errors are calculated by jackknifing across loci. Very few mice were trapped in summer, so those data are not included for most comparisons.

Age	Sex	Season	Location	Relatedness (R)	Std. error	Number of mice
Young	All	Same Spring	Entire grid	0.1148	0.0525	10
Young	Female-female	Same Spring	Entire grid	-0.0131	0.0526	5
Young	Male-male	Same Spring	Entire grid	0.182	0.0924	4
Young	Female-male	Same Spring	Entire grid	0.3362	0.1224	1
Young	All	Same Fall	Entire grid	0.0929	0.0196	37
Young	Female-female	Same Fall	Entire grid	0.0901	0.0249	8
Young	Male-male	Same Fall	Entire grid	0.0902	0.0178	28
Young	Female-male	Same Fall	Entire grid	0.1113	0.0272	9
Young	All	Same Fall	Same trap	0.244	0.0479	14
Young	Female-female	Same Fall	Same trap	0.0206	0.0573	4
Young	Male-male	Same Fall	Same trap	0.3538	0.0851	6
Young	Female-male	Same Fall	Same trap	0.3256	0.044	4
Old	All	Same Spring	Entire grid	0.0492	0.0157	104
Old	Female-female	Same Spring	Entire grid	0.0448	0.0156	34
Old	Male-male	Same Spring	Entire grid	0.0571	0.0154	70
Old	Female-male	Same Spring	Entire grid	0.0439	0.0195	34
Old	All	Same Summer	Entire grid	0.0552	0.0191	19
Old	Female-female	Same Summer	Entire grid	0.0461	0.02	17
Old	Male-male	Same Summer	Entire grid	0.016	0.0209	8
Old	Female-male	Same Summer	Entire grid	0.026	0.0219	14
Old	All	Same Fall	Entire grid	0.0527	0.0242	39
Old	Female-female	Same Fall	Entire grid	0.0735	0.0312	26
Old	Male-male	Same Fall	Entire grid	0.0187	0.0366	13
Old	Female-male	Same Fall	Entire grid	0.0368	0.0347	26
Old	All	Same Spring	Same trap	0.1763	0.0252	41
Old	Female-female	Same Spring	Same trap	0.062	0.0477	6
Old	Male-male	Same Spring	Same trap	0.1654	0.0543	14
Old	Female-male	Same Spring	Same trap	0.209	0.0395	12
Old	All	Same Fall	Same trap	-0.1542	0.082	4
Old	Female-female	Same Fall	Same trap	n/a	n/a	n/a
Old	Male-male	Same Fall	Same trap	-0.231	0.1018	2
Old	Female-male	Same Fall	Same trap	-0.096	0.1257	1

I found no significant correlation between genetic relatedness and geographic distance, except for a very slight positive correlation for both measures of relatedness (Pearson's $r = 0.1$, $p = 0.03$) in Fall 2008. The five females re-trapped in spring presumably overwintered in the field, and were significantly more related to each other than were the five males re-trapped in spring (R-difference = 0.0807 ± 0.04, $p = 0.0001$).

Discussion

Consistent with evidence of high levels of sperm competition in *M. spicilegus* (Gomendio et al. 2006; Gómez Montoto et al. 2011a), I found evidence of multiple paternity in all three litters sampled. As the pregnant females were from two populations over 200 km apart, I do not think that polyandry is simply a rare phenomenon in *M. spicilegus*. Rather, I suggest that polyandry is the norm, as it is also unlikely that males would evolve unusually large testes for their body size without strong selection by polyandrous females. Evidence for multiple mating by females driving the evolution of relatively large testes in males is common in many other socially monogamous species, especially in birds (Birkhead 1987). Interestingly, multiple paternity is most common within species when population densities are high (Westneat & Sherman 1997; Griffith et al. 2002). More samples from a wide geographic range will be necessary to see if *M. spicilegus* is indeed usually polyandrous, and if polyandry is most common when population densities are highest during the breeding season.

In spring and fall, the sex ratios of *M. spicilegus* appear to vary greatly (Table 3.1, Fig. 3.3). Like our results from the trapping grid, another study from Bulgaria in April – May 1992 (Simeonovska-Nikolova 2007) found a male-biased sex ratio in spring. In contrast, a study using exactly the same trapping grid methods found a highly female-biased sex ratio in Hungary April 2000 (Gouat et al. 2003), and another in Bulgaria in May 1994 found no significant sex ratio bias (Belcheva & Metcheva 2001) (Table 1.2). In summer, most reported sex ratios do not appear significantly biased, consistent with our findings from two summers of trapping. However, the sex ratio in fall can be male-biased (Garza et al. 1997; Simeonovska-Nikolova 2000; Poteaux et al. 2008), female-biased (Belcheva & Metcheva 2001; Simeonovska-Nikolova 2007) or even (Simeonovska-Nikolova 2007) like the results

from our trapping grid. I conclude that seasonal sex ratios in *M. spicilegus* are highly variable, and unlikely to be a good indicator of dispersal or mating system in this species unless other ecological factors are known.

Consistent with previous studies (Garza et al. 1997; Poteaux et al. 2008), I show that mice associated with a mound are more related to each other than mice from different mounds in the same population (Fig. 3.5). As predicted, relatedness within mounds is higher in the mound-building season, fall, than in spring, when the mice are thought to disperse to breed (Gouat et al. 2003). This result supports the hypothesis that kinship is one of the ultimate factors explaining the maintenance of mound building as a common good in *M. spicilegus*. There is no significant sex difference in relatedness within mounds at any time of year, suggesting that mound occupation is not driven by kinship between members of a philopatric sex.

Similarly, there is no strong evidence for sex-biased dispersal at the scale of a hectare, as relatedness within the trapping grid was low in all seasons for both males and females. I also re-trapped exactly five females and five males in spring that were first trapped the preceding fall. However, the higher relatedness between the females that remained in the grid after winter, compared to their male counterparts, does suggest that males are more likely to disperse further from their natal territories.

In contrast, results from our trapping grid suggest a strong sex bias in relatedness between mice caught at the same trap in the same season (Fig. 3.6). Not surprisingly, young males associated in space are likely to be close relatives. Our result of low relatedness between

young females from the same trap could be due to the small sample size of four mice. More difficult to explain is the high relatedness between older, sexually mature males from the same trap in spring, when the mice are breeding, and competition for mates should be high. Previous studies did not report catching males at the same trap in spring (Gouat et al. 2003; Simeonovska-Nikolova 2007), but these trapping episodes were half the length of each of our two spring trapping episodes, so the lack of male-male spatial association could be explained by insufficient sampling. More genetic samples would help to determine if the lower female-female relatedness observed in our study is an artifact of trapping fewer females and just chancing upon unrelated females.

Alternatively, I suggest that if polyandry is common in *M. spicilegus*, competition between females should be high, resulting in fewer females being caught at the same trap. In addition, social monogamy is also associated with high levels of inter-female aggression and exclusive female home ranges in mammals (Komers & Brotherton 1997), a pattern that is supported by the high levels aggression between unfamiliar female *M. spicilegus* in a common garden experiment (Dobson & Baudoin 2002). Furthermore, females housed together will reproductively suppress each other (Féron & Gheusi 2003). We do not see lower relatedness between females within the entire grid in any season, only between females at the same trap (Table 3.2), suggesting that the sex-bias in relatedness is not a result of larger-scale sex-biased dispersal. Rather, females seem less likely to associate with close genetic relatives of the same sex, while males have fewer such inhibitions.

A possible explanation for the unexpectedly high relatedness between spatially associated adult males in spring (Table 3.2), is that males benefit by associating with kin, by offsetting

the costs of extra-pair copulations by females through increased inclusive fitness. Increased relatedness between fathers of a litter should reduce the intensity of sperm competition in *M. spicilegus*, which has larger testes relative to body size than the famously promiscuous house mouse (*M. musculus*) (Gomendio et al. 2006). Nevertheless, males that associate socially and spatially with kin can still experience high sperm competition when females are mating multiply, particularly since males in this species participate in paternal care, and invest considerably more in their offspring than sperm alone (Patris & Baudoin 2000; Féron & Gouat 2007).

However a mating system explanation fails to explain the unexpectedly high levels of relatedness between mature males and females at the same trap in spring (Table 3.2). Perhaps these individuals have yet to disperse, and while females are willing to associate with male kin, they tend to avoid female kin. I do not think these patterns of relatedness reflect high levels of inbreeding because of the low levels of population structure reported in chapter 2.

In conclusion, our study shows that while genetic relatedness probably contributes to the maintenance of mound building in *M. spicilegus*, there is no obvious sex bias in the kin composition of mounds, or in natal dispersal in this species. Surprisingly, I find evidence of adult females avoiding female kin, but not male kin, a pattern consistent with social monogamy in mammals, and also with extra-pair paternity, for which I provide the first genetic evidence in this species. High levels of relatedness between adult males in spring suggest that males may be more likely to associate with male kin to offset the costs of being cuckolded.

CHAPTER 4

Coordinated burrowing in *Peromyscus*[1]

Summary

How cooperation in the form of coordinated construction and a division of labor first evolved remains largely unanswered, because the best understood examples involve the most sophisticated instances of cooperation within species. In this study, we ask how social system variation in a group of generally solitary mice in the genus *Peromyscus* is correlated with the capacity for communal construction. By assaying the frequency of burrow cohabitation, burrow length and individual burrowing effort in the lab, we show that while all three species examined are capable of cohabitating and burrowing with an unfamiliar conspecific, the degree of coordination and individual burrowing investment differs among species. Specifically, the two territorial, promiscuous species, *P. leucopus* and *P. maniculatus* only sometimes share a burrow with a stranger, and both same sex and opposite sex pairs of this species fail to dig burrows that are longer than those dug by single individuals. In contrast, the monogamous and less territorial *P. polionotus* invariably shared burrows with unfamiliar, unrelated conspecifics of either sex, and same sex pairs dig significantly longer burrows than individuals, while male-female pairs dig the longest burrows of all. Surprisingly, male *P. polionotus* in pairs invest more in burrowing than females, particularly when paired with an unfamiliar female. We suggest that in this monogamous species, burrowing could be an indicator of male quality driven by female choice for good architects.

[1] This research resulted from a collaboration between Wenfei Tong, Jesse Weber and Zain Ali.

Introduction

Animal architecture provides excellent examples of adaptation, and in many instances, of cooperation involving a division of labor. The canonical examples of eusociality, defined by cooperative brood care, reproductive division of labor and overlapping generations are most famously found in some of the Hymenoptera, termites and naked mole rats (Wilson 1975). However, our detailed knowledge of cooperation in these sophisticatedly social organisms cannot explain how cooperative behaviors such as the communal construction of nests first arise.

Shared construction appears to be an important feature of social evolution in insects (Costa 2006) and rodents (King 1955; Ebensperger 2001; Lacey & Wieczorek 2003; Ebensperger & Blumstein 2006). Extant species of Hymenoptera and rodents can be categorized as falling along a continuum of sociality from solitary to eusocial, and as Darwin proposes in the *Origin*, looking at collateral relatives can offer insights into the early evolutionary stages of the most complex adaptations (Darwin 1859). Solitary halictine bees, for instance, have the capacity to show a division of labor in nest construction when experimentally forced to build in pairs (Jeanson et al. 2005).

Social monogamy with paternal care can be viewed as an instance of reproductive cooperation between two individuals, in which one expects female choice for paternal investment (Trivers 1972). In many birds, males contribute most or all of the nest construction, and in some cases, are chosen by females based on the quality of their nests (Soler et al. 1998b). When both partners contribute to nest construction, architectural

abilities could be a signal of quality in both sexes, and is positively correlated with a larger investment in parental care by both sexes (Soler et al. 1998a).

In this study, we were interested in investigating the capacity for communal construction in a genus of nonsocial rodents, *Peromyscus*, which displays a range of digging behaviors and mating systems (Weber & Hoekstra 2009). In particular, we focused on three species: *P. leucopus*, which facultatively digs burrows, *P. maniculatus*, which regularly digs short burrows, and *P. polionotus*, which digs long burrows and is the only monogamous species of the three (Foltz 1981). Importantly for our study, captive reared *Peromyscus* appear to express their natural burrowing behaviors in the laboratory (Dawson et al. 1988; Weber & Hoekstra 2009).

We were most interested in whether unrelated conspecifics of the same sex would burrow together, and if this capacity for sociality would vary with mating system. As both *P. leucopus* and *P. maniculatus* are territorial and promiscuous (Nicholson 1941; Birdsall & Nash 1972, 1973; Wolff et al. 1983), with high levels of inter-male aggression in *P. leucopus*, we predicted that male-male pairs of these species would be the least likely to burrow together. However, as females of both species occasionally form communal nests with kin (Wolff 1994), we expected female pairs to be more tolerant of one another. Furthermore, some male *P. maniculatus* exhibit paternal care and form pair bonds with females (Wolff & Cicirello 1991), so we predicted that male-female pairs of *P. maniculatus* would be capable of burrowing together.

In contrast with the other two species, *P. polionotus* is both socially and genetically monogamous (Foltz 1981), which led us to predict that female-female pairs of this species would be the least likely to burrow together as inter-female aggression is one of the best predictors of social monogamy in rodents (Komers & Brotherton 1997). We also predicted that this species would put the greatest individual effort into digging when paired with a member of the opposite sex rather than one of the same sex, because of the joint reproductive rewards and mutual signals of quality inherent in building a good burrow. As there is no evidence of sex differences in burrowing in the wild or in the laboratory (Dawson et al. 1988; Weber & Hoekstra 2009), we expected that if coordinated digging took place, there would be an equal investment in digging by both mice in a pair. However, as this is a monogamous species, we did expect the same individuals to invest more in digging when paired with a mouse of the opposite sex, than with a mouse of the same sex. Similarly, we expected long-term, familiar pairs to invest more than unfamiliar male-female pairs.

Methods

Experimental animals and housing

We examined burrowing in three *Peromyscus* species: *P. leucopus, P. maniculatus and P. polionotus*. Mice from all three species are from wild-derived strains from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia, SC, U.S.A.). Each strain has been bred in captivity for 24-61 years, with an effort to minimize inbreeding. Some of the mice were descendants of stock center mice that are now being bred at Harvard University. Before and after all behavioral trials, we housed mice in groups of not more than five individuals of the same sex in 17.78 X 25.4 cm plastic cages containing standard bedding

material and a 5 X 5 cm square of cotton (Ancare Corp., Bellmore, NY, U.S.A.). The ambient temperature was 22°C, and the light cycle 16:8 hours light:dark. We provided standard rodent food and water *ad libitum*. The Institutional Animal Care and Use Committee of the Faculty of Arts and Sciences, Harvard University, approved our animal care standards and experiments (Protocol No. 27-09).

Behavioral assays

We assayed behavior in two different ways, in a room where external visual, olfactory and auditory stimuli were minimized. The first method involved ten chambers of sand (1.22 X 1.52 X 1.07 m), built from 1.27cm thick PVC sheets, with lids consisting of 0.635cm thick metal grating attached to PVC frames. Each chamber was filled with ~1, 000 kg of sand (PharmaServ, MA, USA), after which we shaped the sand into two planes of equal area at different heights (0.85m and 0.40m), connected by a slope of ~60° (Fig. 4.1a**)**. For each trial, we stuck a water bottle to the wall at the lower level, and placed ~5g of rodent food pellets in 5 cm^2 plastic trays on both the higher and lower levels. We also provided nesting material in the form of a 5 cm^2 piece of cotton (Ancare Corp., Bellmore, NY, U.S.A.) near the food tray on the lower level. After each trial, we removed uneaten food, cotton, feces and other debris before wetting and mixing the sand to control moisture and minimize residual scents.

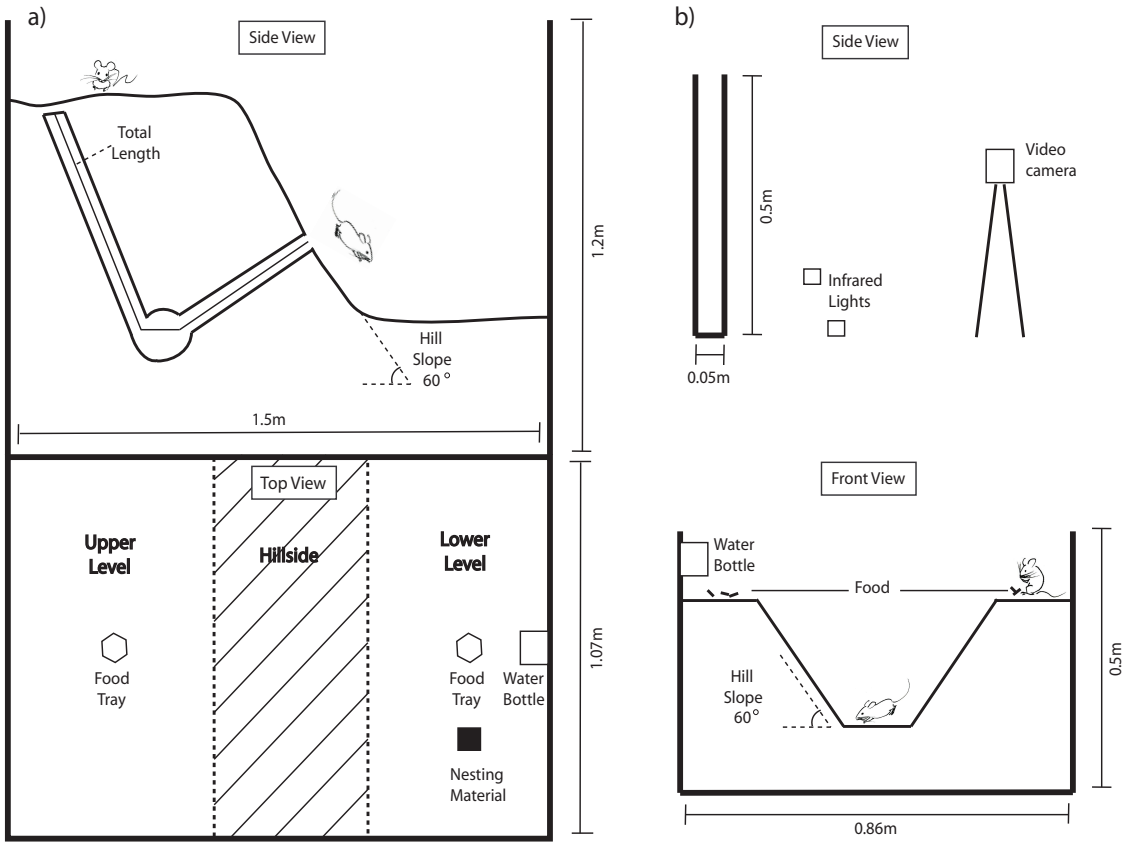


Figure 4.1 Diagrams of both types of burrowing chambers. a) Large sand chambers viewed from the side and from the top, and depicting a typical *P. polionotus* burrow in cross-section, reflecting the way total burrow length is measured. b) Plexiglass "ant farm" video chambers, which are narrow enough to enable us to video burrowing behavior.

For each trial, we introduced either one or two mice to the enclosure between 1600h and 1900h Eastern Standard Time and removed them after approximately 48 hours. In paired trials, we first recorded whether or not mice were occupying the same burrow. In all trials, we measured both the length of the occupied burrow, and the total length of all excavations, as mice would sometimes dig more than one burrow. To minimize chamber effects on burrowing, we switched mice to a different, cleaned sand chamber with each subsequent trial.

Each mouse was tested alone for one to four trials, as well as for two consecutive 48h trials with unfamiliar, unrelated individuals of the same sex, with the exception of a pair of *P.*

leucopus brothers and one of *P. maniculatus* sisters, due to a shortage of animals (Table 4.1). In the paired trials, mice were introduced at the start of, or 48h before a trial. To control for the influence of experience on burrowing behavior, we varied whether mice were tested first as individuals or as pairs. *P. maniculatus and P. polionotus* were also assayed in male-female pairs, with five of the *P. polionotus* pairs initially assayed as pairs, and subsequently, as individuals). Due to a shortage of *P. leucopus*, we did not test male-female *P. leucopus* pairs.

Table 4.1 Summary of behavioral assays in large sand chambers.

I. First tested with a partner then as individuals (2 total trials)

Species	Sex	Sample size
P. leucopus	MM	1
"	M	2
"	FF	-
"	F	-
P. maniculatus	MM	4
"	M	8
"	FF	-
"	F	-
P. polionotus	MM	4
"	M	8
"	FF	4
"	F	8
"	MF	7
"	M	7
"	F	7

II. First tested twice as individuals then twice with a same-sex partner (4 trials)

Species	Sex	Sample size
P. leucopus	MM	4
"	M	8
"	FF	5
"	F	10
P. maniculatus	MM	4
"	M	8
"	FF	5
"	F	9
P. polionotus	MM	2
"	M	5
"	FF	4
"	F	9

III. First tested at least four times as individuals then with a same-sex partner (>5 trials)

Species	Sex	Sample size
P. polionotus	M	8
"	MM	4
"	F	4
"	FF	3

The second testing procedure involved videoing pairs of *P. polionotus* burrowing in narrow plexiglass "ant farms" (Fig. 4.1b). Four chambers of identical dimensions were used for these assays. Each chamber was made from 0.64 cm thick acrylic plexiglass sheets (Plexiglas®) with internal dimensions of (0.86X 0.5 X0.05) m. In each chamber, we used sand (PharmaServ, MA, USA) to form two ~ 60° hills, each ~ 0.25 m high, sloping down to a flat base ~ 0.25 m long. To standardize the moisture content of the sand, both to keep the hills from collapsing, and to standardize burrowing difficulty, we used 3-4 L of water in 16 L of sand for each chamber. Each chamber was supplied with a water bottle and ~ 3g of rodent food. In between trials, we removed all debris and sand that had been in contact with the mice, and let the remaining sand air dry for at least 12h before replenishing the sand and reshaping the hill. We used infrared lights to illuminate the chambers, as mice are most active during their dark cycle, and placed 30mm infrared filters (Opteka, USA) over each video camera lens. The video cameras used were a Sony HandyCam Model No. DCR-SR62 and a Sony HD HandyCam Model No.HDR-XR20.

For each trial, we introduced two mice to an enclosure at 1915 h Eastern Standard Time, just before the start of their dark cycle, and removed them after approximately 15 h. Each trial was videoed for 10-12 h from the moment the mice were introduced to the chambers. Videos were then compressed and converted to mp4 format using the software FFmpeg, and played using VLC media player. We coded the first 10 hours of every video by recording the start and stop times of each digging bout by each mouse. At least 48 h before a trial, we shaved characteristic, identifying patterns on each mouse in order to tell individuals apart in videos. A digging bout is defined as a period in which a mouse displaces sand either by kicking its hind legs, scrabbling with its fore legs, or shoving with its nose for at least 5

seconds. If mice were hidden from view by a thin layer of sand, but we could still see sand flying out of the burrow entrance, we included that time as part of a digging bout.

We had four different treatments: paired adult males and females that had been housed as a pair for at least a year, unfamiliar males and females that were only paired less than 4 h before the start of their first trial, and same sex pairs that were paired for 4 days before their first trial to allow for the mice to acclimatize to each other. For each of the treatments, we tested six pairs of mice twice each, with at least 48h between trials. The same six males and females were used for three different treatments (paired, unfamiliar and same sex), to control for inter-individual differences in burrowing, and six extra mice of each sex were added for the same sex trials.

Statistical analyses

We performed the statistics in R 2.15.1 (R Development Core Team 2012). For the large sand chambers, we tested for an effect of experience on burrowing effort in *P. polionotus*. The other two species were not included in this analysis, as previous evidence showed that both species dig highly consistent burrows across repeated trials (Weber & Hoekstra 2009). We limited our analyses to data collected from mice tested four times as individuals, as it is difficult to evaluate an individual's performance from a paired trial. We regressed the length of the occupied burrow on trial number, and also performed the same regression with the total length of all excavations as the dependent variable.

For all three species of *Peromyscus*, we compared the lengths of occupied burrows in individual to paired treatments (both same and opposite sex pairs). To test for a sex

difference in burrowing either individually or in pairs, we used t-tests. We also used an ANOVA to test for differences between occupied burrow lengths dug by individuals, same sex pairs and opposite sex pairs in all three species, followed by a Tukey post-hoc test to determine the treatments that differed significantly in burrow length.

Finally, we created an "additive ratio" that summarizes the relationship between the length of a burrow dug by a pair to the sum of burrow lengths dug by the same mice as individuals. We used the longest burrows inhabited by pairs, and divided the length of that by the sum of the longest burrow lengths inhabited by each mouse across its individual trials. To test for differences in additive ratio between same and opposite sex pairs within and among species, we performed an ANOVA.

For the video data of *P. polionotus* pairs, we looked at five different measures of individual burrowing effort: individual digging duration, number of digging bouts and proportions contributed by a focal individual to the total paired digging duration, and number of digging bouts. As each individual was tested twice for each treatment, we used Wilcoxon rank-sum tests to check for individuals across the two repeated trials. No significant differences were found, so for each individual, we calculated mean values of the five burrowing effort measures across two trials for each treatment. For each pair, we also calculated means across two trials for the total duration of digging by the pair, and the total number of digging bouts by the pair, as neither measure differed significantly across trials for the same pair of mice.

As with the large sand chamber experiments, we tested for a sex difference in individual contributions for all five individual measures, using Wilcoxon rank-sum tests for

nonparametric data. We also tested for treatment effects on paired burrowing effort in total digging duration and number of digging bouts using one-way ANOVAs. We expected each individual in a pair to contribute half the burrowing, and tested this expectation using a Chi-Square test to see if the proportions of total burrowing time or number of burrowing bouts were significantly different from 0.5 for any of the treatments. We also calculated the between-individual difference in proportion of digging duration and number of digging bouts for each pairing, to see if treatment had an effect on between-individual differences in burrowing effort. If both mice in a pair contributed equally, one would expect the difference in proportions of digging duration and number of digging bouts to be zero. To test for an interaction between the sex of and individual and treatment, we used repeated measures ANOVAs on the six males and six females that were used for familiar and unfamiliar male-female treatments, and for same sex treatments.

Results

Species differences in cohabitation

We found that the three species of *Peromyscus* differ in the frequency of cohabitation of a burrow. As each one pair of *P. leucopus,* three pairs of *P. maniculatus* and twenty pairs of *P. polionotus* were only tested together once, we did not use formal statistical tests, but simply summed the number of times pairs were found occupying the same burrow across all trials (Fig 4.2). *P. leucopus* males occupied the same burrows six out of nine times, and females, in four of ten trials. *P. maniculatus* males cohabitated in seven of twelve trials, while female and opposite sex pairs were always found in the same burrow at the end of every trial. All *P. polionotus* pairs of any combination were found in the same burrow at the end of every trial.

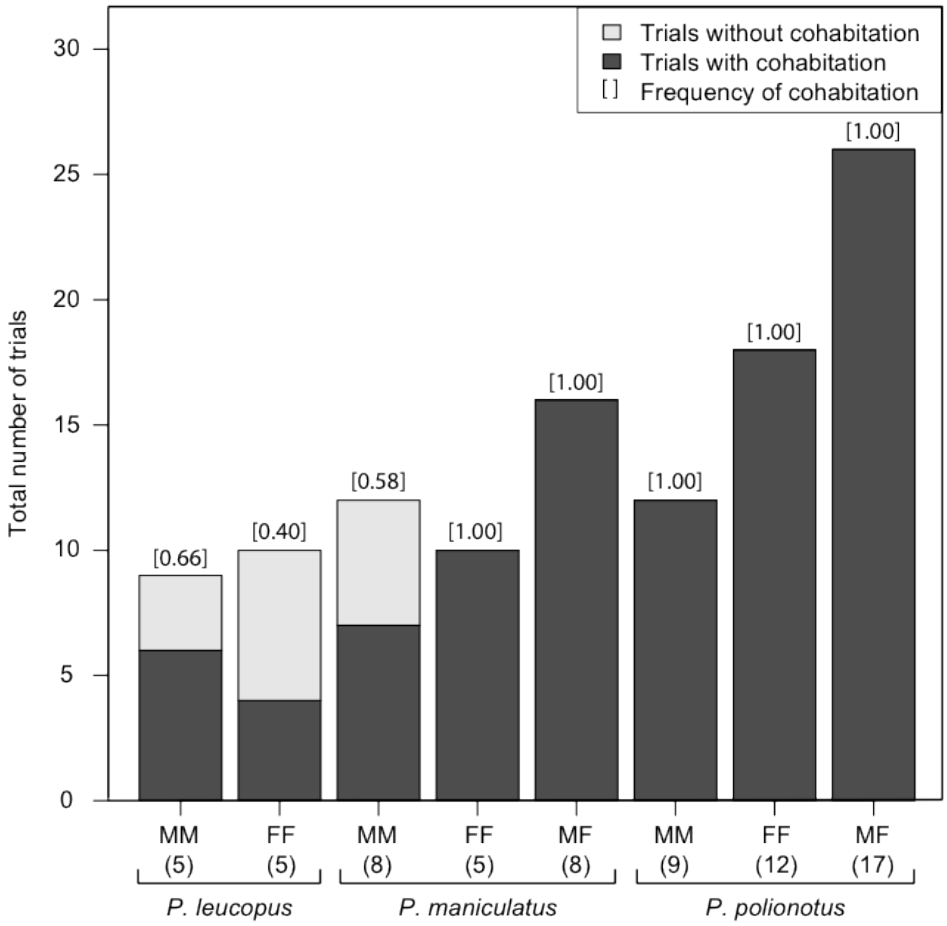


Figure 4.2 Frequency of cohabitation in burrows. We recorded whether two mice were occupying the same burrow at the end of each assay. This figure presents the total number of cohabitation events for both same sex (MM and FF) and opposite sex (MF) pairs, across three *Peromyscus* species. The monogamous *P. polionotus* always cohabitated, regardless of pairing, as did FF *P. maniculatus* pairs. Same sex pairs of *P. leucopus* and MM pairs of *P. maniculatus* cohabitated in approximately half of trials. The number of unique pairs in each treatment are listed in brackets above the species names.

Effects of multiple assays on burrow length

The regression of occupied burrow length on trial was not significant (Fig. 4.3a; df = 1, 46; F = 1.84 $p > 0.05$), but the total length of all excavations in a trial does increase very slightly but significantly with burrowing experience (Fig. 4.3b; df = 1, 46; F=4.41; $p < 0.05$). We also used t-tests to examine the effect of treatment order on burrow length, but found no significant differences (data not reported).

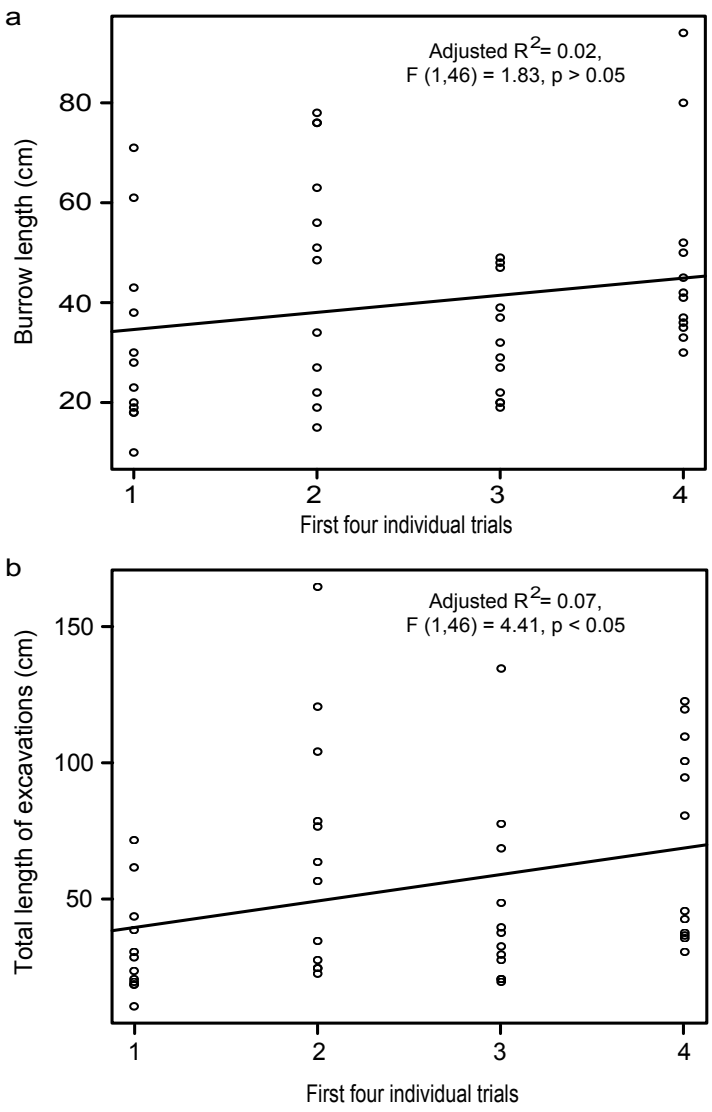


Figure 4.3 Effects of repeated assays on *P. polionotus* burrow length. We tested whether the number of times an individual mouse is tested affects either a) the length of burrows occupied by mice at the end of assays or b) the total length of excavations made during assays. We found no effect of assay number on the length of the occupied burrow, but the length of all excavations increased linearly with assay number.

Burrow length variation

We used the longest burrows occupied by individuals and pairs of mice to test for an effect of treatment on burrow length (Fig. 4.4a). Using t-tests, we found no significant sex

differences in burrow length within species for individuals or pairs. As a result, we grouped same sex pairs and individuals of each species for subsequent analyses.

Using an ANOVA, we showed that there are significant differences in burrow length among treatments and species (Fig. 4.4b; $df = 7, 165$; $F = 31.4$; $p < 0.001$). Post-hoc tests revealed no significant differences in the lengths of burrows dug by individuals or pairs of *P. leucopus* and *P. maniculatus*. *P. polionotus* individuals dig significantly longer burrows than individuals or pairs of the other two species (TukeyHSD $p < 0.001$), and within *P. polionotus*, same sex pairs dig significantly longer burrows than individuals (TukeyHSD $p < 0.05$), while male-female pairs tend to dig longer burrows than same sex pairs (TukeyHSD $p = 0.06$). Burrow length data in each of these treatments are not completely independent, as the same mice were used in individual and paired trials. However, positive correlations between burrowing effort as an individual or pair would make it harder to detect differences, making our results conservative.

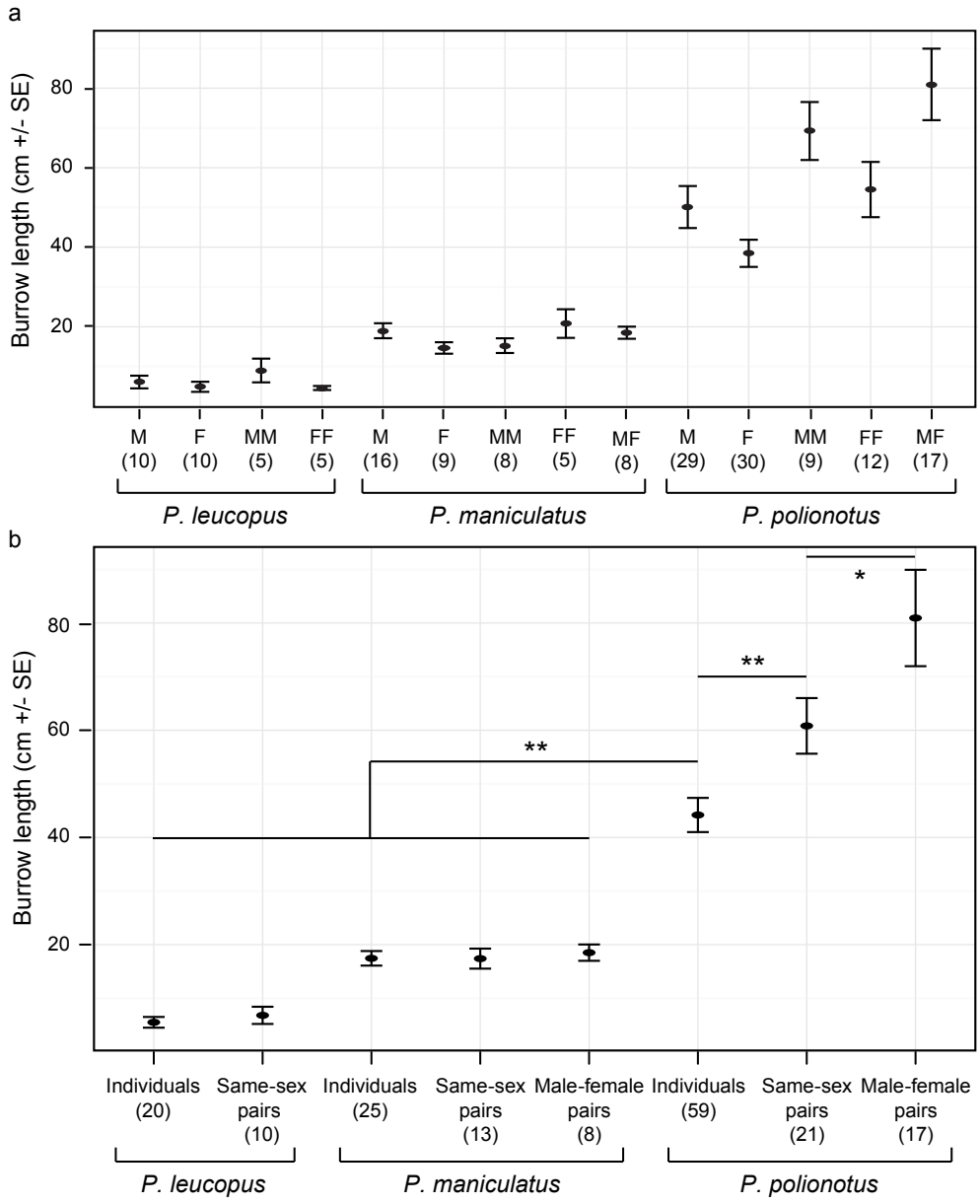


Figure 4.4 Burrow length variation among all treatments. At the end of each trial, we measured burrow length for the burrow(s) that mice were occupying. a) In all three species, there is no significant sex difference for burrows dug individually or in pairs. b) *P. polionotus* individuals built significantly longer burrows than individuals or pairs of mice in other species. Same-sex pairs of *P. polionotus* built significantly longer burrows than individuals, and male-female pairs tended to build longer burrows than same-sex pairs. Sample sizes are listed in parentheses below each group, and significance values are as follows: * indicates $p = 0.06$, ** indicates $p < 0.05$.

Individual contributions to burrow length in paired assays in large sand chambers

Using the "additive ratio" or paired to individual burrow lengths, we performed an ANOVA to test for differences in individual burrowing contributions among same sex and male-female pairs across all three species (Fig. 4.5). The additive ratio varies significantly among groups ($df = 4, 64$; $F = 7.91$, $p < e^{-04}$), and post-hoc tests reveal no significant difference between same sex and male-female pairs of *P. leucopus* and *P. maniculatus*. In contrast to the analysis of burrow length, the additive ratios of same sex pairs of *P. polionotus* do not differ significantly from additive ratios of the other two species. Rather, male-female *P. polionotus* pairs do have a significantly greater additive ratio than any other pairing, including same sex pairs of *P. polionotus* (Tukey HSD $p< 0.01$). Interestingly, the mean additive ratio of male-female *P. polionotus* pairs does not differ significantly from one (one-sample t-test; $df = 17$; $t = 0.34$; $p > 0.73$), which is the expected ratio if both individuals invest the same effort in a shared burrow as they would when digging alone.

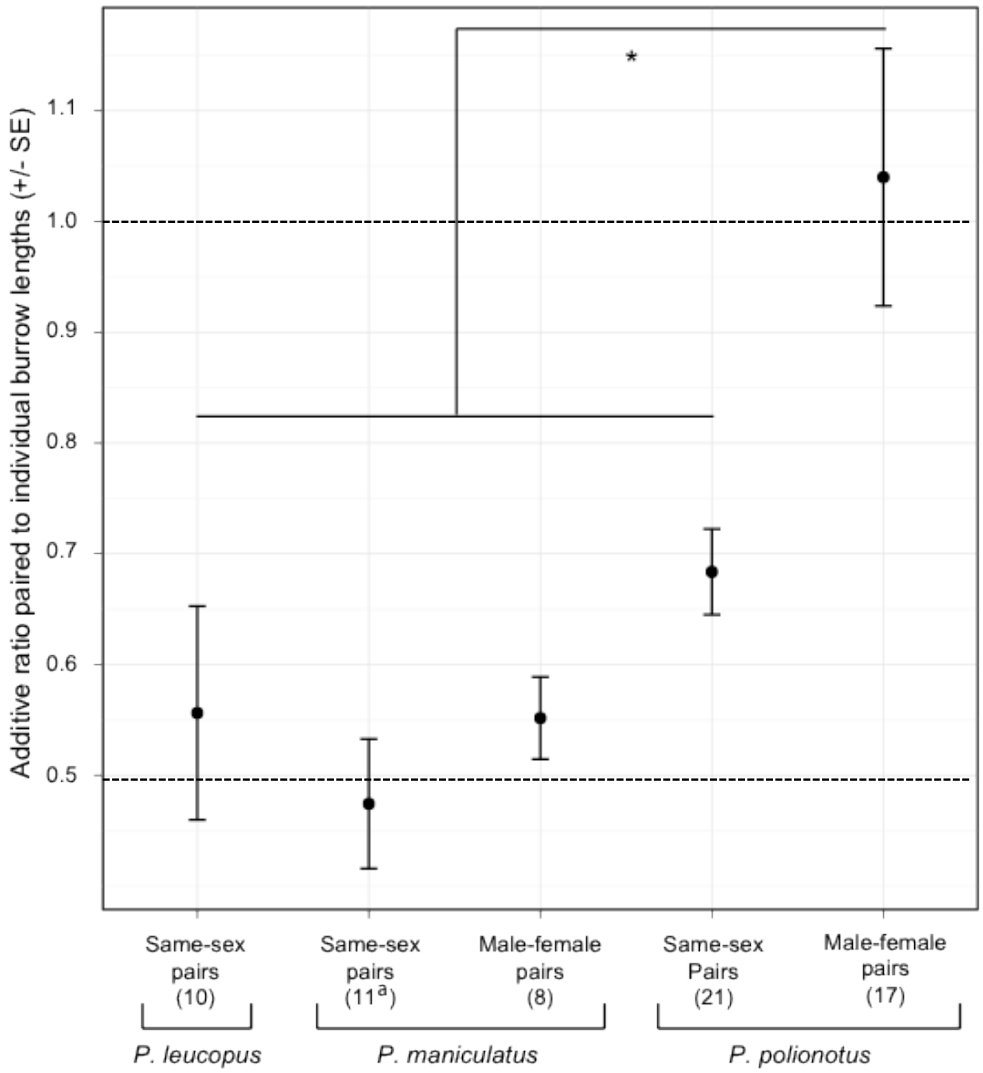


Figure 4.5 Additive ratios of paired to individual burrow lengths. This term directly compares burrow length dug by a pair to the sum of burrow lengths dug by the same mice as individuals. Male-female pairs of *P. polionotus* had a significantly higher ratio than any other group and this ratio was approximately equal to one, suggesting that male-female pairs contribute their full individual efforts when constructing a shared burrow. [a] indicates a difference in sample size between comparisons of absolute burrow length because of lack of individual data.

Individual contributions to burrowing in plexiglass chambers

In contrast to the lack of sex differences in the lengths of burrows dug by individuals or same sex pairs of *P. polionotus* in the large sand chambers, we found that across all pairings in the plexiglass chambers, females dig less than males for all metrics except mean bout duration, for which there is no significant sex difference (Fig. 4.6, Appendix Fig. 1).

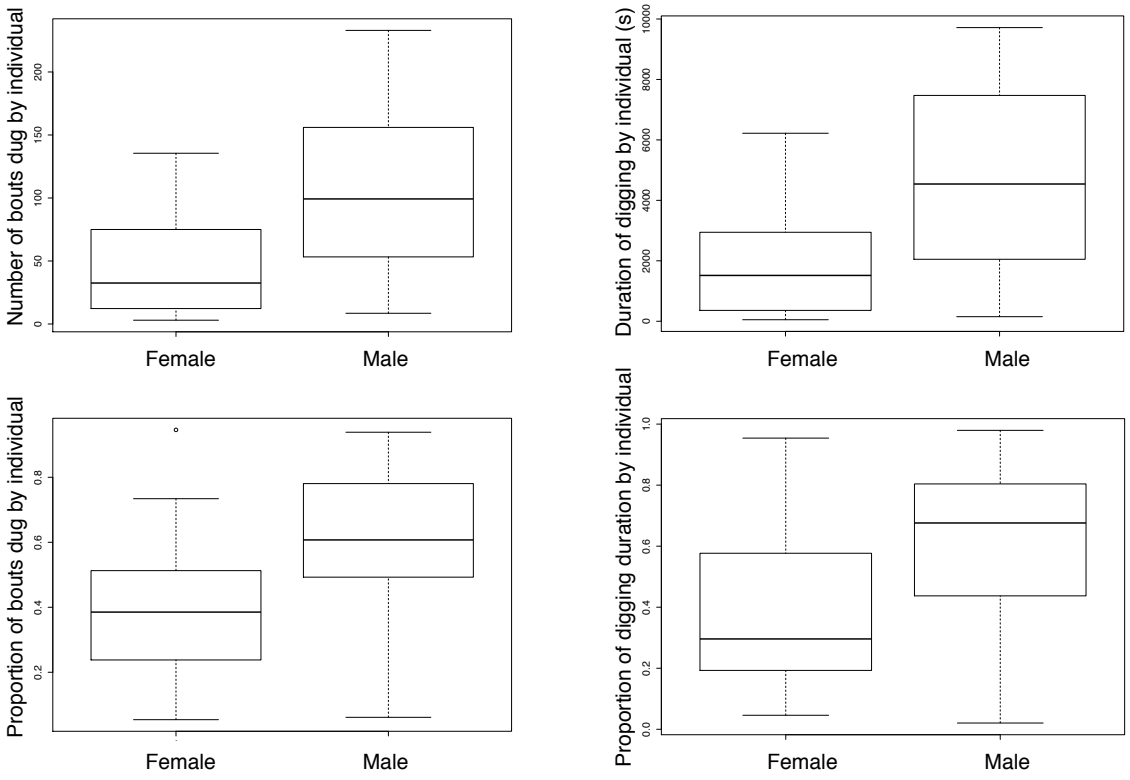


Figure 4.6 Boxplots of four different measures of individual burrowing effort across all three different paired treatments. Females burrow significantly less than males in terms of both absolute values and proportion of the paired totals for number of digging bouts and total digging duration in seconds.

More intriguingly, we found an effect of treatment on number of digging bouts ($df = 1, 10$; $F = 7.55$, $p < 0.05$), individual digging duration ($df = 1, 10$; $F = 10.27$, $p < 0.01$), proportion of digging bouts ($df = 1, 10$; $F = 18.14$, $p < 0.01$) and proportion of digging duration ($df = 1, 10$; $F = 15.82$, $p < 0.01$). Post-hoc tests showed that females paired with another female dig fewer times than when paired with familiar males (paired t-test: $n= 6$, $p = 0.02$). Similarly, the total digging duration of a female is lower when she is paired with another female, than when she is paired with either a familiar (paired t-test: $n= 6$, $p = 0.02$) or unfamiliar male (paired t-test: $n= 6$, $p = 0.01$) (Fig. 4.7).

A one-way ANOVA revealed no significant differences among treatments for paired digging duration or number of digging bouts. However unpaired t-tests do show that female-female pairs dig fewer times than male-male pairs ($p = 0.03$), and spend less time digging in total ($p = 0.01$). In addition, unfamiliar male-female pairs, but not familiar male-female pairs dig fewer times in a trial than two males ($p = 0.03$).

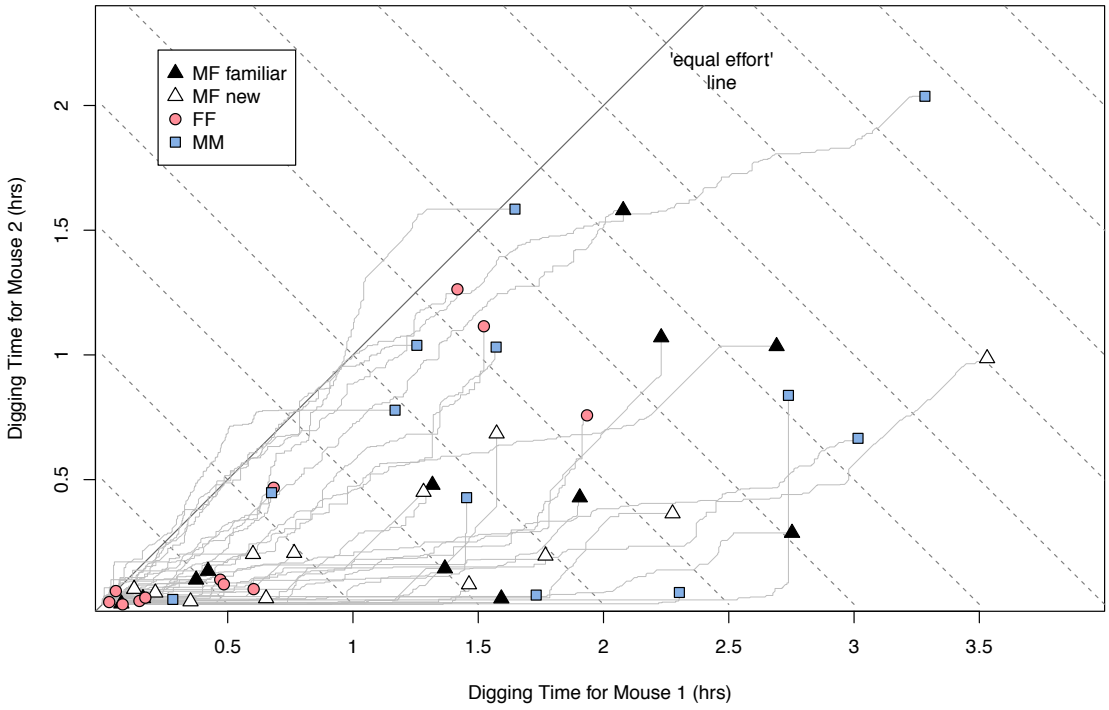


Figure 4.7 Plot showing the asymmetry in digging contributed by mice in a pair. On the horizontal axis is time spent digging by males in all male-female pairings, and by the mouse that dug more in same-sex pairs. On the vertical axis is time spent digging by females in male-female pairs, or the mouse that dug less in a same-sex pair. Each point represents a pair of mice, with total time spent digging by each individual. Grey traces leading to the shape track the contributions of each mouse in the pair over the course of a trial. The "equal effort line" represents all values at which both mice would have dug for an equal length of time. Dotted grey lines represent a particular total digging time, so all points that fall on the same dotted line are pairs that dug for the same total duration. All pairs fall below the "equal effort line", but with more same-sex pairs near it than male-female pairs. Unfamiliar male-female pairs fall furthest from the "equal effort line". The pairs that spent the greatest total time digging are almost all male-male pairs and familiar male-female pairs, while pairs that spent the least total time digging are mostly female-female pairs and unfamiliar male-female pairs.

For none of the four treatments did individual contributions to total digging by either sex depart significantly from 0.5 (Chi-Square test, $p > 0.9$). Nevertheless, female pairs do have a significantly smaller between-individual difference in the proportion of digging bouts contributed than do unfamiliar male-female pairs (t-test; $p = 0.03$). In addition, unfamiliar male-female pairs show a greater between individual difference in both proportion of digging bouts and the proportion of digging time than male-male pairs (t-test; $p < 0.05$), implying that males split the burrowing more equally with male partners than with novel female partners (Fig. 4.7).

Discussion

As predicted, *P. leucopus* of both sexes are least likely to share a burrow with an unfamiliar conspecific of the same sex. Similarly, *P. maniculatus* males did not always share burrows with each other. That *P. maniculatus* did, is consistent with the fact that females of this species occasionally breed communally (Wolff 1994). Nevertheless, we were surprised these two territorial species were willing to spend any time at all with unfamiliar members of the same sex. Also contrary to our expectations, the monogamous *P. polionotus* invariably cohabitated with unfamiliar and unrelated members of either sex. Perhaps this result is less surprising in light of detailed field studies of this species, demonstrating that adults of the Alabama beach mouse subspecies (not used in this study) frequently share overlapping ranges, and display low levels of aggression towards neighbors, who are likely to be relatives because of unusually low dispersal distances (Tenaglia et al. 2007). There is also evidence of a low frequency (5%) of burrow cohabitation between members of the same sex in the wild (Rand & Host 1943; Foltz 1981). One explanation for the unexpectedly high frequency of burrow cohabitation between unfamiliar and unrelated individuals, is that the mice we tested were

generally housed in groups of five from the age of weaning, and may not have been exposed to social conditions necessary for territoriality to develop. More interestingly, there could actually be an adaptive benefit to sharing a burrow, even with conspecifics of the same sex. In the termite *Reticulitermes speratus*, unrelated females need a partner to successfully found a colony, possibly because reciprocal allogrooming is necessary for new queens to survive numerous pathogens (Matsuura et al. 2002).

Both individuals and conspecific pairs of all three species constructed burrows comparable to previous laboratory and field studies (Dawson et al. 1988; Weber & Hoekstra 2009). The burrow lengths we observed are consistent with the hypothesis that the monogamous species is most likely to show an increase in burrowing investment by both individuals when paired with a member of the opposite sex. Only male-female *P. polionotus* pairs invested their full individual efforts in digging a joint burrow, whereas neither *P. leucopus* nor *P. maniculatus* pairs dug significantly longer burrows than individuals. A proximate explanation is that both these species are programmed to dig till their burrow has reached a predetermined length, at which point burrowing stops. Such behavior is consistent with the hypothesis that a burrow is merely a place to hide, and once an animal is out of sight, it stops digging, a pattern observed in Mongolian gerbils (Wiedenmayer 1997). Interestingly, *P. polionotus* pairs of the same sex also dug significantly longer burrows than individuals, although these burrows were not as long as those dug by male-female pairs. Two studies have also reported suggestive evidence of coordinated burrowing by unrelated *P. polionotus* pairs of the same sex (Rand & Host 1943; Wolfe & Esher 1978). In summary, our results from measuring burrow lengths in the large sand chambers show no significant sex difference in any of the species, and an increase in total burrow length in paired *P. polionotus* relative to individuals, but not in the

other two species. Most interestingly, the additive ratio is close to one only for male-female *P. polionotus* pairs, suggesting that only in male-female pairs of the monogamous species will both individuals contribute their full individual digging efforts towards a shared burrow.

To test the hypothesis that *P. polionotus* males and females contribute equally and fully to a shared burrow only when paired with a member of the opposite sex, we quantified burrowing from video footage. To our surprise, female *P. polionotus* showed a striking lack of digging in contrast to their male counterparts, and in spite of showing no significant sex difference in burrow length as individuals or same sex pairs measured in large sand chambers, or in the wild (Dawson et al. 1988; Weber & Hoekstra 2009). A simple explanation for this difference is simply that females are more disturbed by the smaller plexiglass chambers, and dig less than they would in other situations, whereas males are less bothered by the plexiglass environment. In addition, the small sample sizes and relatively inbred mice used could result in an artificial sex difference that is not reflected in more outbred wild populations. Alternatively, there could be a previously unappreciated sexual division of labor in *P. polionotus*. Consistent with this interpretation is our observation from videos (data not shown) that females tend to spend more time excavating a nest cavity once the majority of the burrow is complete. A more detailed quantification of nesting and burrowing behavior, preferably involving a large number of more outbred mice would help to distinguish potential artifacts from promising evidence of a division of labor between the sexes in burrow construction by a monogamous mammal.

Our results also suggest that individuals modify their digging effort conditionally, depending on the sex and familiarity of their partner. Females paired with both unfamiliar and familiar

males dig significantly more than when paired with another female. A proximate explanation for this result is that mice are inspired to burrow more if exposed to a partner who burrows a lot. An ultimate explanation for the same result could be that females invest more in burrows that are shared with males because those are most likely to lead to shared reproductive benefits.

Two mice of the same sex show the smallest difference between individuals in digging effort. However the only significant result is that males paired with an unfamiliar female dig significantly more than their partner, compared to males paired with another male. The smaller inequality between two males can easily be explained by the fact that males are always motivated burrowers in these assays, so two males are far more likely to dig equal amounts than a male and a female. However, it would be interesting to explore the gradation in inequality of digging effort between males paired with familiar and unfamiliar females. If a larger sample size also showed a significant difference in digging inequality between males paired with familiar and unfamiliar females, this would suggest that burrowing in *P. polionotus* is an indicator of male quality before pair bonding occurs in this monogamous species.

In summary, *Peromyscus* species vary in their capacity for coordinated construction and a division of labor between the sexes, with the most monogamous species showing the greatest mutual investment in joint burrowing, especially when paired with the opposite sex. We suggest that this pattern is consistent with burrowing as a sign of male quality, and that the least territorial *P. polionotus* is most pre-adapted to group living and burrowing.

CONCLUSION

The two very different wild mice studied in this dissertation provide some clues to the causes and consequences of cooperative construction. The low level of population differentiation across the species range in *M. spicilegus* suggests that in spite of gathering in genetically related groups to build mounds in fall, this species could disperse widely enough to maintain gene flow across distances of over 200km. Alternatively, the low levels of population differentiation could be the result of a bottleneck before the species colonized its currently geographically disjoint range in Europe.

There is no strong evidence of sex-biased gene flow in *M. spicilegus*, and similarly no sex-bias in the kinship structure of mounds. However we do find an excess of adult males in spring 2008, and a higher pairwise relatedness between males from the same trap than between females from the same trap. One explanation for this pattern is that at high densities, males preferentially associate with kin to minimize the costs of extra-pair paternity. Based on data from three litters collected from two populations over 200km apart, this study does confirm that the socially monogamous *M. spicilegus* does indeed engage in extra-pair copulations.

Like all cooperative relationships, monogamous pairs engaged in parental care are vulnerable to conflict. In the case of *M. spicilegus*, we expect males to engage in mate guarding, but behavioral observations of nocturnal mice are difficult to procure. In response to extra-pair copulations, males can also evolve to produce more sperm, a prediction well-supported by the relatively large testes in *M. spicilegus* that led to our hypothesis that this species is not genetically as well as socially monogamous.

Like *M. spicilegus*, *Peromyscus polionotus* is also socially monogamous with paternal care. However *P. polionotus* is also largely genetically monogamous, and members of both sexes appear to be less territorial than *M. spicilegus*. As some of the pre-conditions for eusociality are monogamy and delayed dispersal and the shared construction of shelters, we expect *P. polionotus* to have the capacity to evolve into a cooperatively breeding species under ecological conditions in which resources are more clumped in space and time. In the meantime, this study does show that *P. polionotus* has the capacity to burrow in a coordinated manner with a conspecific, and that there is a division of labor between the sexes, with males doing most of the digging. One explanation for this sex difference is that monogamous females have selected for males that are motivated diggers.

In both *M. spicilegus* and *P. polionotus*, shared construction and parental care by both sexes constitute examples of cooperation with individual conflicts of interest. While kinship is probably a factor influencing cooperative construction in *M. spicilegus*, genetic relatedness is not necessary for coordinated burrowing in *P. polionotus*, a situation more compatible with a snowdrift game. Similarly, shared parental care in both monogamous mice takes place between unrelated individuals with conflicts of interest over who should invest more care in the current offspring, at the expense of additional individual reproduction. As shared shelters and social monogamy are often associated with cooperative breeding, both mouse species can offer insights into the transition from communal construction to cooperative breeding.

Appendix

Table 1. Table of DNA sources, including museum and GenBank accession numbers, and full list of sample IDs corresponding to haplotype names.

Country	Locality	Latitude °N	Longitude °E	N	mtDNA haplotype	Sample ID	GenBank	Microsatellite group
Austria	Waldacker	47.939214	16.97134	3	AHS1	MVZ192270 MVZ192278 MVZ192279	This report	
				1	AHS2	MVZ192277		
Austria	Halbturn	47.858324	16.97525	1	AHS1		U47536	
Austria	Mönchhof	47.88211	16.94231	2	A1	MM283 MM282	EU106300 EU106299	
Hungary	Unknown	Unknown	Unknown	2	H1	H043 H045	This report	3 (15)
				1	H2	H044		
				1	AHS1	H036		
				2	AHS2	H016 H041		
Hungary	Kápolna	47.759113	20.24708	25	AHS1	H015 H055 H104 H106 H107 H108 H110 H114 H115 H116 H117 H118 H119 H120 H121 H122 H123 H124 H125 H126 H127 H129 H130 H131 H132	This report	5 (65)
				3	H3	H111 H112 H113		
				6	AHS2	H011 H013 H014 H049 H105 H109		

Table 1 (Continued). Table of DNA sources, including museum and GenBank accession numbers, and full list of sample IDs corresponding to haplotype names.

Hungary	Sződ	47.724664	19.17139	7	H3	H021 H022 H023 H028 H030 H037 H042	This report	4 (47)
				5	H4	H035 H040 H047 H048 H054		
				1	H5	H038		
				1	H6	H027		
				16	AHS2	H017 H018 H020 H024 H025 H026 H029 H032 H033 H034 H039 H046 H050 H051 H052 H053		
Slovakia	Komárno-Ďulov Dvor	47.78647	18.16795	13	AHS1	MM1338 MM1340 MM1341 MM1342 MM1343 MM1344 MM1345 MM1346 MM1347 MM1348 MM1349 MM1350 MM1352	This report	2 (15)
Slovakia	Vrbová nad Váhom	47.849314	18.05090	3	AHS1	MM1336 MM1337 MM1335	EU106306 EU106307 This report	1 (3)

Table 1 (Continued). Table of DNA sources, including museum and GenBank accession numbers, and full list of sample IDs corresponding to haplotype names.

Slovakia	Kechnec	48.549383	22.26444	39	AHS2	17119 17124 17125 17126 17128 17293 17294 17295 17296 17299 17300 17301 17302 17303 17304 17305 17306 17307 17310 17311 17312 17313 17314 17315 17316 17425 17438 17747 17748 17749 17750 17751 17752 17753 18574 18681 18704 18705 18706	This report	6 (40)
				1	Slo1	17309		
Slovakia	Belža	48.580792	22.27416	3	AHS2	16848 16849 16850	This report	7 (3)
Serbia	Debeljača	45.066667	20.6	1	Se4	162501	This report	
Serbia	Pančevo	44.855868	20.69824	1 1 1	Se1 Se2 Se3	MM739 MM741 MM742	EU106308 EU106309 EU106310	
Bulgaria	StrainZBN	Unknown	Unknown	1	B5		AB039263	
Bulgaria	Srebarna	44.094442	27.06402				This report	8 (34)
Bulgaria	Knezha	43.497984	24.08117				This report	12 (86)
Bulgaria	Telish	43.327022	24.26103	3	B1	B2.3.1 B2.7.1 B2.7.2	This report	11 (224)
				3	B2	B2.2.1 B2.2.2 B2.2.4		
				2	B3	B2.6.1 Te62		
Bulgaria	Krushovitsa	43.348975	24.41527	6	B1	B2.4.1 B2.4.2 B2.4.3 B2.4.4 B2.7.1 B2.8.1	This report	9 (7)
				1	B2	B2.3.1		
Bulgaria	Rakita	43.285017	24.23035	1	B4	R059	This report	10 (31)

Table 1 (Continued). Table of DNA sources, including museum and GenBank accession numbers, and full list of sample IDs corresponding to haplotype names.

Moldova	Kishinev	47.085085	28.78417	1 1 1	Mol1 Mol2 Mol3	 MM226 MM227	U47537 EU106321 EU106322	
Ukraine	Dshankoi	45.708611	34.39333	1	U1		U47538	
Montenegro	Ulcinj	42.929722	19.22429	1 1 2	Mon1 Mon2 Mon3	3775 3776 3793 3794	EU106311 EU106312 EU106313 EU106301	
Greece	Igoumenitsa	39.50615	20.26553	1 1	G1 G2	BG4677 BG4678	EU106314 EU106315	13 (2)
Greece	Vlaherna	39.172623	20.99896	1 1 1	G5 G4 G3	BG4653 BG4657 BG4654	EU106316 EU106317	14 (2)
Greece	Komeno	39.046958	22.03213	2	G3	MM1227 MM1228	EU106302 EU106303	15 (2)
Greece	Patras1	38.249626	22.73545	3	G6	MM1215 MM1216 MM1217	EU106318 EU106304 EU106305	16 (5)
Greece	Patras2	38.266469	22.74992	1 1	G7 G8	MM1218 MM1226		16
Greece	Rozena	38.119795	22.39723	1	G9		EU626226	
Greece	StereaEllada	38.604393	22.71521	2	G10		EU626224 EU626225	

Figure 1. Individual plots for each videod trial of P. poliontus pairs digging. Each mouse represented by a line showing an increase in cumulative digging time over the course of a 10 hour trial. Males are in blue, females in pink. Black lines represent the sum of individual digging times. At the top of each plot, colored bars represent intervals when an individual mouse is digging, making it easy to see when two mice are burrowing simultaneously. This first page shows plots for all the familiar male-female pairs.

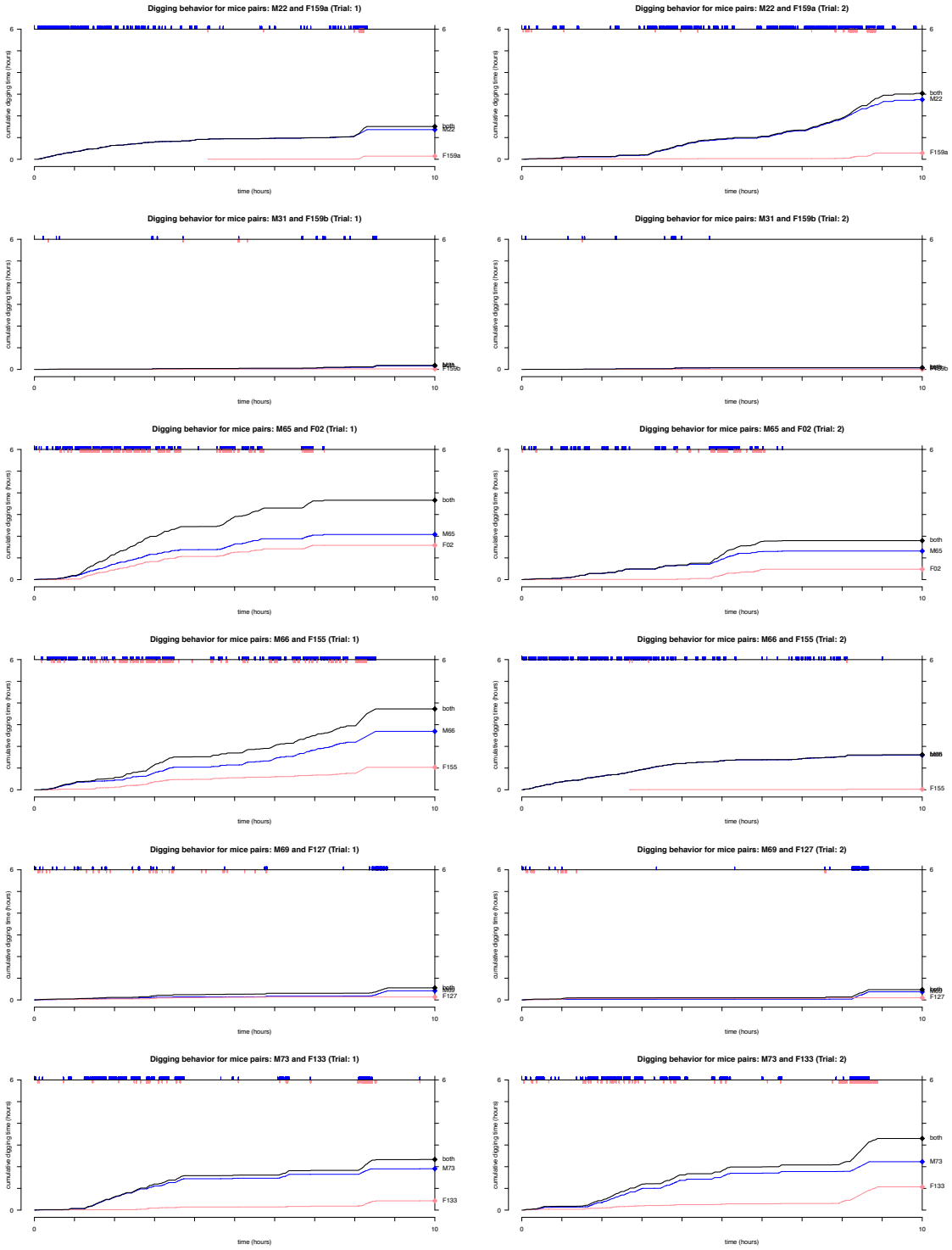


Figure 1 (Continued). Plots of digging over time for unfamiliar male-female pairs.

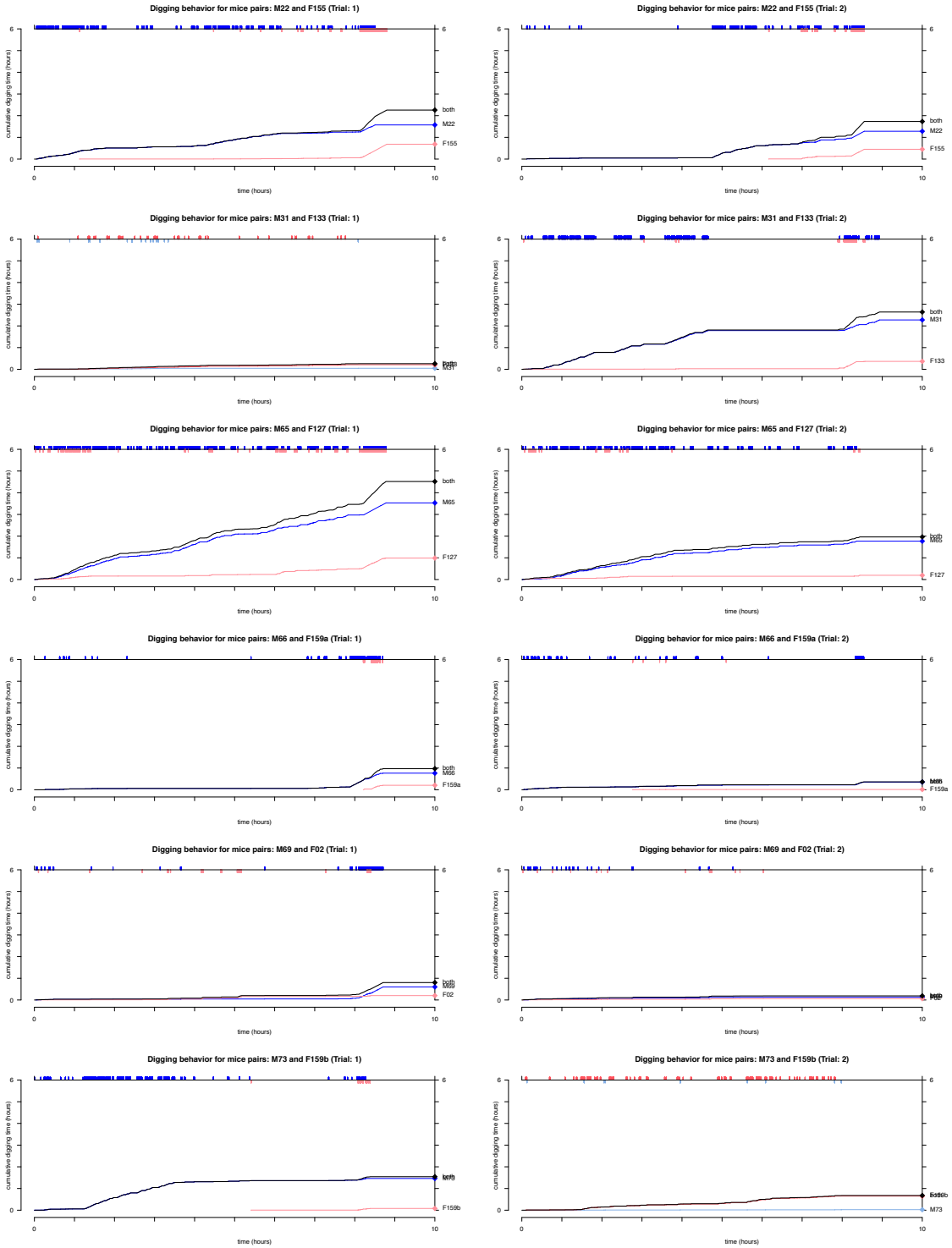


Figure 1 (Continued). Plots of digging over time for female-female pairs.

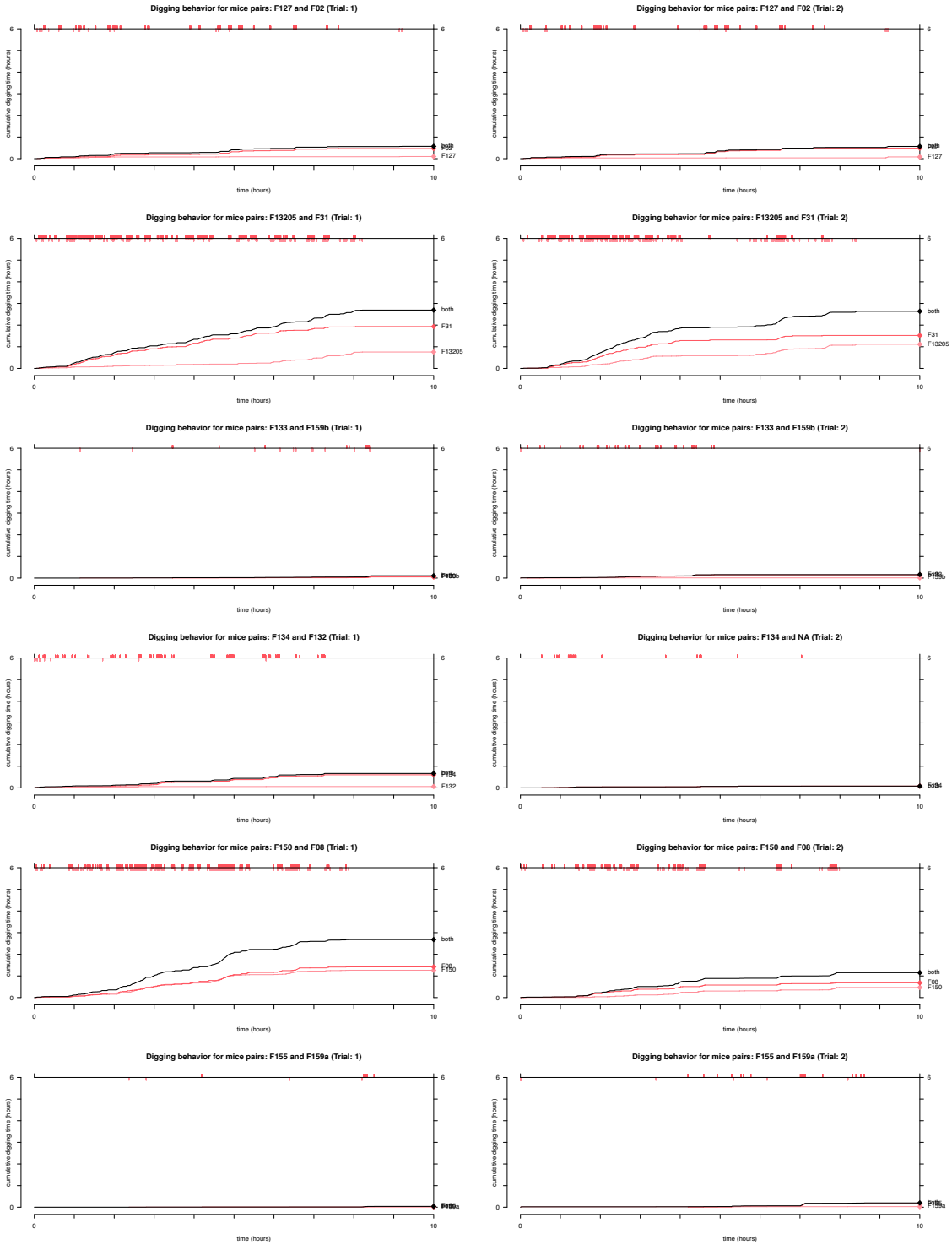
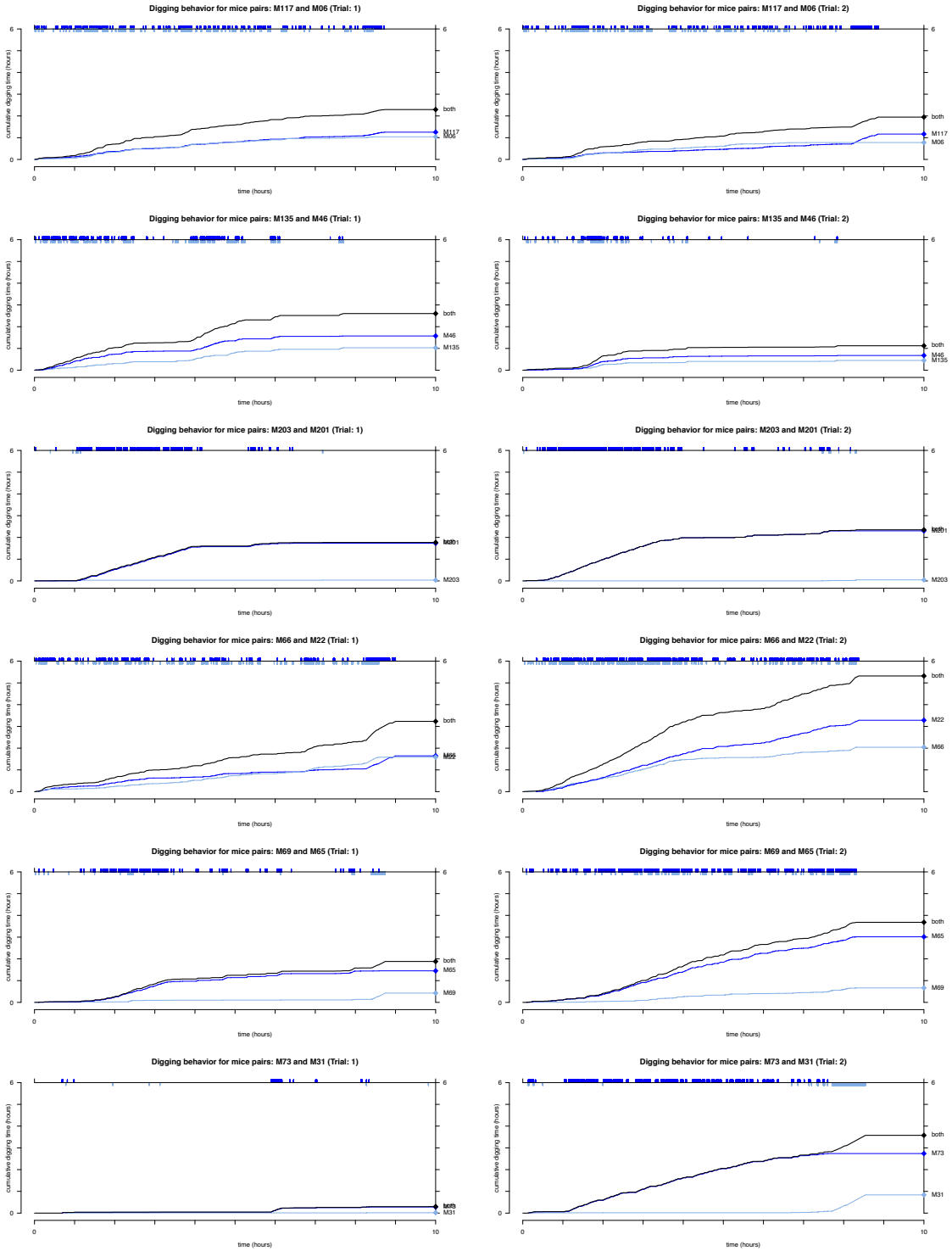


Figure 1 (Continued). Plots of digging over time for male-male pairs.



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